

PA 1186642

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

June 24, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/505,638

FILING DATE: September 25, 2003

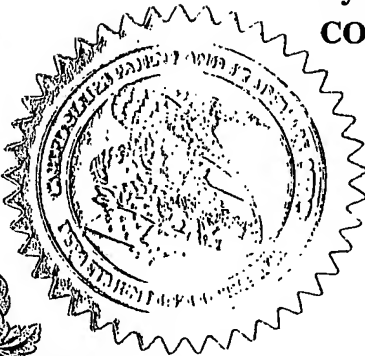
REC'D 12 AUG 2004

WIPO

PCT

**PRIORITY DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH  
RULE 17.1(a) OR (b)

By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS



*T. Wallace*  
T. WALLACE  
Certifying Officer

BEST AVAILABLE COPY

PATENT APPLICATION SERIAL NO. \_\_\_\_\_

U.S. DEPARTMENT OF COMMERCE  
PATENT AND TRADEMARK OFFICE  
FEE RECORD SHEET

09/30/2003 SNIWASS1 00000022 60505638

01 FC:2005

80.00 DP

PTO-1556  
(5/87)

Gary M. Nath (DC, NJ)  
Harold L. Novick (DC, MD)  
Todd L. Juneau (DC, IL)  
Ross A. Epstein (CA)\*  
Marvin C. Berkowitz (GA)\*  
Robert C. Ryan (NY, IL)\*

Irvin A. Lavine (DC), Retired  
Donald M. Sandler (MD), Retired\*

Patent, Trademark and Copyright Causes  
Unfair Competition, Trade Secrets,  
Licensing and Litigation

\*Practice limited to Matters and Proceedings before  
Federal Courts and Agencies; not Admitted in DC

**NATH & ASSOCIATES PLLC**  
Attorneys at Law  
1030 Fifteenth Street, N.W.  
Sixth Floor  
Washington, D.C. 20005-1503

TELEPHONE (202) 775-8383  
(202) 775-9393

FACSIMILE (202) 775-8396  
(202) 822-9409

E-MAIL: IP@NATHLAW.COM  
WEB: WWW.NATHLAW.COM

Michelle L. Harland (VA)\*  
Lee C. Heiman (CA)\*  
Jerald L. Meyer (VA)\*  
Tanya E. Harkins (MD)\*  
Joshua B. Goldberg (VA)\*  
Sheldon M. McGee (DC)\*  
Derek Richmond (VA)\*  
Alvin E. Tanenholtz (VA)\*  
Angela Y. Dai

\*\* Registered Patent Agent; not Admitted in DC  
++ Limited Rep. at USPTO

60/5055638

**COVER SHEET FOR FILING U.S. PROVISIONAL APPLICATION**  
**UNDER 37 CFR 1.53(c)**

Commissioner of Patents and Trademarks  
Alexandria, Virginia 22313-1450

Re: New U.S. Provisional Patent Application  
For: **SPHINGOLIPIDS' POLYAMINES CONJUGATES  
MEDIATED DELIVERY SYSTEMS**  
Inventors: **Elimelech ROCHLIN; D.N. Shimshon,  
ISRAEL  
Yechezkel BARENHOLZ; Jerusalem,  
ISRAEL**  
Attorney Docket: 25733

Sir:

Attached hereto is the application identified above, includ-  
ing:

72 Pages Application Consisting of:  
31 pages Textual Specification,  
4 pages of claims,  
1 page of the abstract,  
9 pages of tables,  
27 sheets drawings;

— Executed Inventor's Declaration

The present provisional application names the following  
inventor(s): 1) Elimelech ROCHLIN; D.N. Shimshon, ISRAEL  
2) Yechezkel BARENHOLZ; Jerusalem, ISRAEL

The Government filing fee\* is calculated as follows:  
Base Fee (Provisional Application) . . . . . \$ 160.00

**TOTAL FILING FEE\***  
(accounting for possible small entity status) . . . \$ 80.00

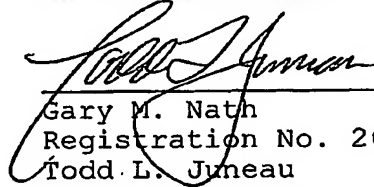
X "Reduced by one-half, as applicant(s) is/are a "small entity".

27 Sheets of Drawing(s) is/are attached.

X Submitted herewith is a check in the amount of \$ 80.00. The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,

**NATH & ASSOCIATES PLLC**

  
\_\_\_\_\_  
Gary M. Nath  
Registration No. 26,965  
Todd L. Juneau  
Registration No. 40,669  
Customer No. 20529

Date: September 25, 2003

**NATH & ASSOCIATES PLLC**

1030 15<sup>TH</sup> Street NW - 6<sup>th</sup> Floor

Washington, D.C. 20005

(202)-775-8383

GMN/TLJ/lis (ProvisionalAppl.coversheet)



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Elimelech ROCHLIN et al.

Serial No. NOT YET ASSIGNED

Filed: September 25, 2003

For: SPHINGOLIPIDS' POLYAMINES CONJUGATES MEDIATED DELIVERY  
SYSTEMS

TRANSMITTAL LETTER

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

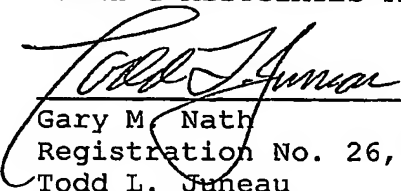
Submitted herewith for filing in the U.S. Patent and Trademark  
Office is the following **PROVISIONAL APPLICATION**:

- (1) Transmittal Letter
- (2) Cover sheet for filing Provisional Application;
- (3) 72 page Provisional Application consisting of:
  - 31 pages Textual Specification,
  - 4 pages of claims,
  - 1 page of the abstract,
  - 9 pages of tables,
  - 27 sheets drawings;
- (4) Check No. 1944 \$ 80.00 for filing fee as a small entity;
- (5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or  
credit any excess to Deposit Account No. 14-011.

Respectfully submitted,  
NATH & ASSOCIATES PLLC

By:

  
\_\_\_\_\_  
Gary M. Nath  
Registration No. 26,965  
Todd L. Juneau  
Registration No. 40,669  
Customer No. 20529

Date: September 25, 2003  
NATH & ASSOCIATES PLLC  
1030 15<sup>TH</sup> Street, NW - 6<sup>th</sup> Floor  
Washington, D.C. 20005  
GMN/TLJ/lg:APPL.trans

## **SPHINGOLIPIDS' POLYAMINES CONJUGATES MEDIATED DELIVERY SYSTEMS**

5

### **FIELD OF THE INVENTION**

The present invention concerns vehicles for effective delivery of biologically active agents, particularly polynucleotides, proteins, peptides, and drug  
10 molecules, by facilitating transmembrane transport or by encouraging adhesion to biological surfaces and/or affecting intracellular traffic and processing of the biologically active agent as well as affecting the cells interacting by the delivery system to better respond biologically.

### **LIST OF PRIOR ART**

- 15 1. US 5,334,761
2. US 2001/048939
3. US 5,659,011
4. US 5,674,908
5. US 5,910,306
- 20 6. US 5,980,898
7. Lopes de Menezes DE, Hudon N, McIntosh N, Mayer LD. 2000. Molecular and pharmacokinetic properties associated with the therapeutics of bcl-2 antisense oligonucleotide G3139 combined with free and liposomal doxorubicin. Clin. Cancer Res 6:2891-2902.
- 25 8. Jansen B, Wacheck V, Heere-Ress E, Schlagbauer-Wadl H, Hoeller C, Lucas T, Hoermann M, Hollenstein U, Wolff K, Pehamberger H. 2000.

Chemosensitization of malignant melanoma by BCL2 antisense therapy. *Lancet* 356:1728-1733.

9. Chi KN, Wallis AE, Lee CH, Lopez de Menezes D, Sartor J, Dragowska WH, Mayer LD. 2000. Effects of Bcl-2 modulation with G3139 antisense oligonucleotide on human breast cancer cells are independent of inherent Bcl-2 protein expression. *Breast Cancer Res Treat* 63:199-212.

10. Guinness ME, Kenney JL, Reiss M, Lacy J. 2000. Bcl-2 antisense oligodeoxynucleotide therapy of Epstein-Barr virus-associated lymphoproliferative disease in severe combined immunodeficient mice. *Cancer Res* 60:5354-5358.

11. Waters S, Webb A, Cunningham D, Clarke PA, Raynaud F, di Stefano F, Cotter FE. 2000. Phase 1 clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 18:1812-1823.

12. Gao X, Huang L. (1999) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochim. Biophys. Acta* 179: 280-285.

13. Max-Delbruck-Centrum für Molekulare Medizin: WO 98/05,678 (1998).

14. Miller AD. (1998) Cationic liposomes for gene therapy. *Angew. Chem. Int. Ed. Eng.* 37: 1768-1785.

## BACKGROUND OF THE INVENTION

20 Many natural biological molecules and their analogues, including proteins and polynucleotides, foreign substances and drugs, which are capable of influencing cell function at the sub-cellular or molecular level are preferably incorporated within the cell in order to produce their effect. For these agents the cell membrane presents a selective barrier which is impermeable to them. The  
25 complex composition of the cell membrane comprises phospholipids, glycolipids, and cholesterol, as well as intrinsic and extrinsic proteins, and its functions are influenced by cytoplasmic components which include  $Ca^{++}$  and other metal ions, anions, ATP, microfilaments, microtubules, enzymes, and  $Ca^{++}$ -binding proteins.

Interactions among structural and cytoplasmic cell components and their response to external signals make up transport processes responsible for the membrane selectivity exhibited within and among cell types.

Successful intracellular delivery of agents not naturally taken up by cells has also been investigated. The membrane barrier can be overcome by associating agents in complexes with lipid formulations closely resembling the lipid composition of natural cell membranes. These formulations may fuse with the cell membranes on contact, and in these processes, the associated substances are delivered intracellularly (adsorptive endocytosis).

Lipid complexes can facilitate intracellular transfers also by overcoming charge repulsions between the cell surface, which in most cases is negatively charged, and the molecule to be inserted. The lipids of the formulations comprise an amphipathic lipid, such as the phospholipids of cell membranes, and form various layers or aggregates such as micelles or hollow lipid vesicles (liposomes), in aqueous systems. The liposomes can be used to entrap the substance to be delivered within the liposomes; in other applications, the drug molecule of interest can be incorporated into the lipid vesicle as an intrinsic membrane component, rather than entrapped into the hollow aqueous interior, or electrostatically attached to aggregate surface.

An advance in the area of intracellular delivery was the discovery that a positively charged synthetic cationic lipid, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of liposomes, or small vesicles, could interact spontaneously with DNA to form lipid-DNA complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in both uptake and expression of the DNA (Felgner, P. L. et al. Proc. Natl. Acad. Sci., USA 84:7413-7417 (1987) and U.S. Pat. No. 4,897,355 to Epstein, D. et al.). Others have successfully used a DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) in

combination with a phospholipid to form DNA-complexing vesicles. The Lipofectin<sup>TM</sup> reagent (Bethesda Research Laboratories, Gaithersburg, Md.), an effective agent for the delivery of highly anionic polynucleotides into living tissue culture cells, comprises positively charged DOTMA liposomes which interact  
5 spontaneously with negatively charged polynucleotides to form complexes. When excess of positively charged liposomes over DNA negative charges are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces or introduced into the cells either by endocytosis or fuse with the plasma  
10 membrane, both processes deliver functional polynucleotide into, for example, tissue culture cells.

Multivalent cations by themselves (including polyamines, inorganic salts and complexes and dehydrating solvents) have also been shown to facilitate delivery of macromolecules into cells. In particular, multivalent cations provoke the  
15 collapse of oligo and polyanions (nucleic acids molecules, amino acid molecules and the like) to compact structural forms, and facilitate the packaging of these polyanions into viruses, their incorporation into liposomes, transfer into cells etc. (Thomas T.J. et al. Biochemistry 38:3821-3830 (1999)). The smallest natural polycations able to compact DNA are the polyamines spermidine and spermine. By  
20 attaching a hydrophobic anchor to these molecules via a linker, a new class of transfection vectors, the polycationic lipopolymers, has been developed.

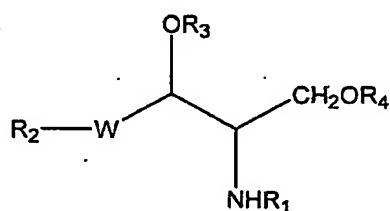
Cationic lipids and cationic polymers interact electrostatically with the anionic groups of DNA (or of any other polyanionic macromolecule) forming DNA-lipid complexes (lipoplexes) or DNA-polycation complexes (polyplexes).  
25 The formation of the complex is associated with the release of counterions of the lipids or polymer. The cationic lipids can be divided into four classes: (i) quaternary ammonium salt lipids (e.g. DOTMA (Lipofectin<sup>TM</sup>) and DOTAP) and phosphonium/arspium congeners; (ii) lipopolyamines; (iii) cationic lipids bearing

both quaternary ammonium and polyamine moieties and (iv) amidinium, guanidinium and heterocyclic salt lipids.

## SUMMARY OF THE INVENTION

The present invention is based on the fact that novel compounds may serve as efficient vehicles for the delivery of polynucleotides, oligonucleotides, proteins, peptides and drugs into cells by facilitating their transmembrane transport.

Thus the present invention is directed to a compound of formula (I):



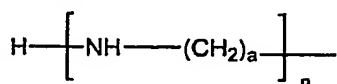
wherein

$\text{R}_1$  is selected from hydrogen, branched or normal alkyl, aryl, lower alkyl amine wherein said amine may be substituted, or  $\text{C}(\text{O})\text{R}_5$

$\text{W}$  is  $-\text{CH}=\text{CH}-$ ,  $-\text{CH}_2-\text{CH}(\text{OH})-$  or  $-\text{CH}_2-\text{CH}_2-$ ,

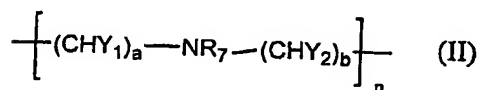
$\text{R}_2$  and  $\text{R}_5$  are independently selected from a saturated or unsaturated normal or branched  $\text{C}_{10}-\text{C}_{24}$  alkyl, alkenyl or polyenyl groups, preferably from  $\text{C}_{12}-\text{C}_{18}$ ; and most preferably  $\text{C}_{12}-\text{C}_{16}$ ;

$\text{R}_3$  and  $\text{R}_4$  are independently selected from hydrogen, a group  $-\text{C}(\text{O})-\text{NR}_6\text{X}-\text{Z}$ ,  $\text{Z}$  being a same or different for  $\text{R}_3$  and  $\text{R}_4$ ; or  $\text{R}_3$  and  $\text{R}_4$  form together with the oxygen atoms to which they are bound a heterocyclic group comprising  $-\text{C}(\text{O})-\text{NH}-\text{Z}-\text{NH}-\text{C}(\text{O})-$ ;  $\text{R}_6$  is hydrogen or



wherein  $a$  represents an integer from 1 to 4;  $n$  represents an integer from 1 to 6 preferably from 1 to 3;

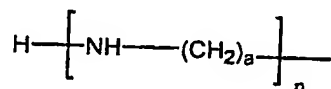
Z being a branched or linear polyalkylamine chain wherein said polyalkylamine chain may comprise quaternary ammonium groups; with the proviso that R<sub>3</sub> and R<sub>4</sub> are not simultaneously hydrogen. The polyalkylamine chain is of formula (II):



wherein a and b are independently selected from 1, 2, 3 or 4;

n represents an integer from 1 to 6, preferably from 2 to 4 ;

R<sub>7</sub> is selected from hydrogen or



where a and n have the meaning as above; and Y<sub>1</sub> and Y<sub>2</sub> are independently selected from hydrogen and alkyl.

Lower alkyl is defined as a C<sub>1</sub>-C<sub>5</sub>-alkyl group which may be linear or branched and which may substituted.

The present invention is further directed to a process for preparing a compound of formula (I).

The invention is further directed to a method for introducing biologically active compounds into eukaryotic cells comprising forming liposomes from the compound of formula (I), contacting said liposomes with a biologically active compound to form a complex and administering said complex.

The invention is yet further directed to a complex comprising liposomes formed from the compound of formula (I) and a biologically active compound. The biologically active compound is selected from polynucleotides, oligonucleotides, proteins, peptides and drugs. Administration of polynucleotides and oligonucleotides may be used in gene therapy, gene vaccination and transfection. Proteins, peptides and drugs may be used in new methods of vaccination and drug delivery.

The invention is yet further directed to pharmaceutical compositions comprising the complex comprising liposomes formed from the compound of formula (I) and a biologically active compound together with suitable excipients. The pharmaceutical composition may be in the form suitable for intravenous, subcutaneous, topical, intranasal, oral, ocular, intramuscular administration.

### BRIEF DESCRIPTION OF THE FIGURES

In order to understand the invention and to see how it may be carried out in practice, some embodiments will now be described, by way of non-limiting examples only, with reference to the accompanying figures, in which:

10 Fig. 1 shows several possible chemical structures, "linear", branched" or "cyclic" compounds according to the present invention.

Fig. 2 Shows the Mass Spectrum of a specific compound (3-amino-propyl) - [4-(3-amino-propylamino)-butyl]-carbamic acid -hexadecanoylamino-3-hydroxy-octadec-4-enyl ester (chemical structure shown). The Expected M.W. is 766.  
15 Elementary analysis: C - 6.5%; H-11.65%, N-8.01%. TLC system: Chloroform:Methanol: Acetic acid 1:25:1.5. TNBS-reactive primary amines: 2 per molecule. Solubility: ethanol (base).

Fig. 3 shows growth of MCF-7 cells following application of (A) LUV (large unilamellar vesicles)-derived or (B) HV(heterogeneous vesicles)-derived  
20 lipid-like cationic (LLC)- assemblies. Throughout, [S-ODN] = 0.1  $\mu$ M and [L+]/[S-ODN] = 2. Each error bar represents the s.e.m. where n (no of wells)  $\geq$  9. The data were derived from experiments performed on 3 separately cultivated cell batches.

Fig. 4 shows Western blots for quantification of bcl-2 in MCF-7 cells. (A)  
25 Bcl-2 western blot in MCF-7 cells following application of various treatments. (B) Corresponding densitometric analysis of bcl-2 western blot in MCF-7 cells.



Fig. 5 shows MCF-7 cell growth as a function of Genofect<sup>TM</sup> lipoplex (HV-derived) concentration. Throughout,  $[L+]/[ODNP-] = 2$ . Each error bar represents the s.e.m. where  $n$  (no of wells)  $\geq 9$ . The data were derived from experiments performed on 3 separately cultivated cell batches.

5 Fig. 6 shows MCF-7 cell growth as a function of lipid-like (LLC) lipoplex (HV-derived) charge ratio. Throughout,  $[S-ODN] = 0.1 \mu M$ . Each error bar represents the s.e.m. where  $n$  (no of wells)  $\geq 9$ . The data was derived from experiments performed on 3 separately cultivated cell batches.

Fig. 7 shows toxicity studies on NIH 3T3 cells revealing that the lipid-like  
10 cationic (LLC) vehicles (B5C - certain batch in the form of a chloride salt) are non toxic to cells.

Fig. 8 shows gene transfection of luciferase plasmid into NIH 3T3 cells. Transfection was carried by lipid-like cationic (LLC) vehicles together with DOPE.

Fig. 9 shows a comparison of transfection of C-26 cells with luciferase: a  
15 comparison between lipid-like cationic (LLC) vehicles and FuGENE6®.

Fig. 10 shows a pictorial comparison of the efficiency of transfection of pGFP into cells using the lipid-like cationic (LLC) vehicles of the present invention.

## DETAILED DESCRIPTION OF THE INVENTION

20 The present invention concerns novel vehicles for delivering of polynucleotides, oligonucleotides, proteins, peptides and drugs into cells. The vehicles are lipid-like cationic (LLC) compounds, which may be synthesized in the following manner. N-substituted long-chain bases in particular, N-substituted sphingoids or sphingoid bases are coupled together with different polyamines or  
25 their derivatives, to form a polyamine-sphingoid entity, which is used as is or further alkylated. Protonation at a suitable pH or alkylation of the formed polyamine-sphingoid entity attributes to the lipid-like compounds the desired positive charge for interaction with both the active biological entities to be

delivered into the target cells and with the targeted cells. The formed lipid-like compounds, after their synthesis may be directly complexed efficiently with biological entities in the form of anions or polyanions to form complexes (lipoplexes). Alternatively, prior to their complexation they are converted into vesicles. Non limiting examples of such biological entities are polynucleotides, oligonucleotides, proteins, peptides and drugs. Thus the formed complex is suitable for targeting such active biological entities into cells. Non-limiting examples of the sphingoids or sphingoid bases are sphingosine, dihydrosphingosine, phytosphingosine, dehydrophytosphingosine or derivatives thereof. Non-limiting examples of such derivatives are acyl derivatives, such as ceramide (N-acylsphingosine), dihydroceramides, phytoceramides and dihydrophytoceramides, respectively. The suitably N-substituted sphingoids or sphingoid bases, in addition to the free amine which may be derivatized, possess free hydroxyl groups which are activated and subsequently reacted with the polyamines to form the polyamine-sphingoid entity. Non-limiting examples of activation agents are N,N'-disuccinimidylcarbonate, di- or tri-phosgene or imidazole derivatives. The reaction of these activation agents with the sphingoids or the sphingoid bases yields a succinimidylloxycarbonyl, chloroformate or imidazole carbamate, respectively, at one or both hydroxyls. The reaction of the activated sphingoids with polyamines may yield branched, normal (unbranched) or cyclic compounds. Fig. 1 displays chemical structures of several possible compounds prior to their quaternization. Fig. 2 further displays the Mass Spectrum of one specific compound, ceramide coupled with spermine. Formation of branched, normal or cyclic polyamine sphingoid may be directed by monitoring the excess of polyamine used in the reaction and suitable protection of polyamine prior to use. The formed conjugates of the sphingoids with the polyamines could be further reacted with methylation agents in order to form quaternary amines. The resulting compounds are positively charged to a different degree depending on the ratio between the quaternary, primary and/or secondary amines within the formed

conjugates. The resulting non-methylated or methylated lipid-like compounds may then be processed by any known method to form liposomes. Such processing may include (non-limiting examples) incorporation of different non-cationic lipids like DOPE, Cholesterol or others at different mole ratios to the lipid-like compound.

5 The formed liposomes may be shaped as heterogeneous vesicles (HV) having a diameter of about 500 - 5000 nm. The formed HV, may be downsized and converted to large unilamellar vesicles (LUV) having a diameter of about 100 nm by further processing. The structure and dimensions of the vesicles, e.g. their shape and size may have important implications on their efficiency as vehicles for

10 delivery of the active biological entities to the target, i.e. these determine their transfection properties. Thus the structure of the formed vesicles, HV or LUV, is one important factor. Another important factor for efficient delivery is the ratio between the amount of the amine positive charge of the lipid-like ( $L^+$ ) and the negatively charged oligo or polyanion complexed therein (or on the lipid-like

15 surface) ( $A^-$ ). The ratio determines the overall charge of the charged complex, where for effective delivery the ratio may be  $0.1 < L^+/A^- < 1000$  depending on the entrapped moiety. In case of a particular oligonucleotide of the present invention, the ratio was found to be  $1 < [L^+/\text{ODN}] < 2$ .

Such transfection facilitates for example mucosal vaccination, introduction

20 of genes into cells for their expression, gene and oligo- and poly - nucleotide therapy. In addition, the lipid-like cationic liposomes are able to efficiently induce on mice peritoneal macrophage surface the expression of high levels of MHC II and costimulatory molecules such as B7 and CD40. These are essential for productive antigen presentation. Neutral and anionic liposomes do not possess such

25 abilities. This can explain the unique and superior adjuvant activity of the formulations of the present invention. Thus the formed complexes may be part of a pharmaceutical composition comprising the complex bearing the active biological entity to be delivered together with suitable excipients. Such a pharmaceutical

composition may be prepared such that it may be administered intravenously, subcutaneously, topically, intranasally, orally, ocularly or intramuscularly.

One important example of efficient use of the lipid-like cationic (LLC) vehicles is in oligonucleotide transfer into cells, and in particular into cancerous  
5 cells. One approach in cancer treatment is to target specified poly- or oligonucleotides in the form of antisense in order to interfere with cancer cell function. Many types of tumors overexpress bcl-2, a key apoptosis regulating protein that has been implicated in mechanisms of chemoresistance by blocking programmed cell death. An 18-mer antisense phosphorothioate oligonucleotide (S-ODN) targeted to  
10 the first 6 codons of the open reading frame of bcl-2 was designed (Genta Inc.). In order to achieve efficient treatment, the antisense oligonucleotide should be transferred into cancerous cells. Such targeting requires an appropriate vehicle. Commercial lipids and lipid-like compounds synthesized according to the present invention were used in order to find the optimal complex, which delivers the  
15 S-ODN in a most efficient manner. DOTAP, DOPE, DOPC, cholesterol and their mixtures were used to form lipoplexes and liposomes. These commercial liposomes and lipoplexes were compared with the lipid-like compounds according to the present invention. Fig. 3 shows the results of experiments performed with lipid-like cationic lipoplexes and liposomes based on commercial lipids. As can be seen  
20 from Fig. 3A, when MCF-7 breast cancer cells were incubated with LUV-derived lipid assemblies, cell growth was either unaffected or very marginally reduced. Fig. 3B presents the influence of HV-derived complexes and vesicles. In this case, treatment with all LLC/DOPC assemblies, or all LLC/DOPE assemblies or LLC liposomes induced a negligible effect on cell growth. However, while incubation  
25 of MCF-7 cells with LLC-reverse sequence (of the antisense 18-mer) lipoplexes produced a marginal (14%) reduction in cell growth, incubation with LLC-S-ODN complexes decreased cell growth to a large extent (50%). Moreover, this difference was highly significant (Student's t-test:  $P < 0.001$ ). Confirmation of the direct involvement of bcl-2 in cell growth inhibition was accomplished by the use of a

Western blot technique to quantify bcl-2 protein expression. Fig. 4A and Fig. 4B, respectively, show the Western blot results and the corresponding bcl-2/actin ratio values derived from densitometric analysis. The densitometry clearly reveals that while incubation with S-ODN- LLC lipoplexes reduced cellular bcl-2 levels by some 90%, incubation with the other control treatments *i.e* LLC liposomes, reverse sequence- LLC lipoplexes or free S-ODNs, exerted a negligible or marginal influence on cellular bcl-2 levels. These results indicate that the delivery of S-ODN as MLV-derived LLC lipoplexes, facilitates enhanced antisense downregulation of bcl-2. In fact, by using this lipid vector with 0.1  $\mu$ M G3139, cell growth was reduced by approximately the same extent as using free G3139 at a concentration of 5  $\mu$ M (data not shown). Thus, antisense efficacy was improved 50-fold by using the LLC lipoplex of the present invention.

The efficiency of the LLC/S-ODN depends on the effective concentration of LLC/S-ODN and on ratio between the S-ODN and LLC entity. Since the LLC is positive, while the S-ODN (like any polynucleotide) is negatively charged, the latter concerns the charge balance of the formed complex.

In order to determine the concentration dependence, lipoplexes were prepared at a fixed  $L^+$  to  $ODNP^-$  mole ratio (charge ratio) of 2. The data is presented in Fig. 5. It can be seen that at an S-ODN concentration of 0.05  $\mu$ M, all the lipid assemblies exerted a negligible or marginal effect on cell growth. However at a concentration of 0.2  $\mu$ M, exposure to both S-ODN lipoplexes and reverse sequence lipoplexes suppressed cell growth. This indicates that enhanced antisense activity by these complexes develops only at a very narrow ODN concentration "window" around 0.1  $\mu$ M. If the concentration of complexes is slightly reduced below this value, the complexes exert no effect while if complex concentration is slightly elevated above this value they exert a non-specific cellular toxicity. In order to ascertain the optimal ratio between the S-ODN and its vehicle, concentration was maintained at 0.1  $\mu$ M and MCF-7 cell growth was assessed as a function of the  $L^+$  to S-ODN (which is actually S- $ODNP^-$  since the phosphate

groups of the oligonucleotide determine its negative charge) mole ratio of HV-derived lipid-LLC assemblies. It can be seen from Fig. 6 that only complexes formed at  $L^+ / ODNP^-$  mole ratio of 1 or 2 facilitated enhanced antisense efficiency. More positively charged or negatively charged lipoplexes were ineffective in  
5 downregulating bcl-2.

Gene transfection into cells is another utilization of the lipid-like vehicles of the present invention. Fig. 7 clearly demonstrates that the LLC vehicles of the present invention (in particular, the chloride salt of positively charged amine groups) are not toxic when transfected into NIH 3T3 cells. Turning to Fig. 8,  
10 effective transfection of luciferase into NIH 3T3 cells is demonstrated. The vehicle used was a mixture of the LLC vehicles together with the commercial lipid DOPE. A comparison of transfection into C-26 cells using LLC vehicles and FuGENE6® is shown in Fig. 9. Fig. 10 further shows a pictorial demonstration of the efficiency of transfection of pGFP into NIH 3T3 cells using a mixture of LLC vehicles/DOPE  
15 in a 2:1 ratio.

Another use of the lipid-like cationic vehicles is in vaccination, in particular mucosal vaccination. Although most pathogens use the mucosal routes (respiratory, gastrointestinal, urogenital) for invasion, the majority of the currently available vaccines are administered parenterally, usually i.m. Parenteral  
20 immunization has been found to be very effective in evoking the systemic immune response, however, local immunity at the portals of pathogen entry is often suboptimal with injectable vaccines. In contrast, potent mucosal vaccines can trigger both local and systemic immunity, and mucosal immunity may be less affected by aging compared with systemic immunity. Moreover, since needle-free  
25 mucosal vaccines can be self-administered, compliance would be greater, and large populations could be immunized within a short period of time.

A major problem with mucosal (e.g., intranasal, oral) vaccines consisting of killed microorganisms or their purified products is poor immunogenicity. Unless fortified with strong adjuvants, such vaccines may even induce tolerance.

Unfortunately, some of the most powerful adjuvants in animals, such as cholera toxin (CT) or the *E. Coli* enterotoxin are too toxic and are not approved for human use.

The immunostimulating activity of antigen-loaded liposomes is attributed to:

- 5 (a) protection of the entrapped antigen against degradation or neutralization, (b) slow release of the antigen over an extended period of time (depot effect), and (c) efficient uptake by antigen-presenting cells (APC) such as dendritic cells and macrophages. Two injectable liposomal vaccine formulations, against hepatitis A and influenza, are already in use in humans.

- 10 The lipid-like cationic vehicles of the present invention, when loaded with the appropriate antigen(s) and optionally with an adjuvant and administered intranasally (i.n.), are highly effective in inducing strong humoral and cellular, local and systemic immune responses, and protective immunity, against the influenza virus. Intranasal administration of the loaded lipid-like cationic liposomes elicited  
15 robust local (in the nose and lungs) and systemic (in the serum) hemagglutination inhibition (HI), IgG1, IgG2a and IgA responses, cytotoxic activity, IFN $\gamma$  production, and protective immunity. Cationic formulations, composed of both commercially known and the lipid-like cationic vehicles of the present invention, when given i.n., were up to 10,000 times more efficacious than the non-liposomal  
20 vaccine or liposomal vaccine formulations comprising neutral and anionic lipids.

In order to evaluate the optimal composition most efficient for vaccination, various liposomal vaccine formulations were compared for antigen encapsulation efficiency and immunogenicity, using varying lipid/antigen w/w ratios (with and without addition of cholesterol) and instillation volumes, and with and without an  
25 adjuvant. In addition, the induction of humoral and cellular responses by various routes (intranasal, intramuscular, oral) and the effect of each route on the immune response and protective immunity to the live virus challenge were also tested. The results are given in Tables 1-8.

### HN Antigen encapsulation

Efficiency of HN (a commercial preparation of hemagglutinin and neuraminidase derived from influenza A virus) encapsulation in the various liposomal formulations at different lipid/protein w/w ratios (3/1-300/1), and with  
5 and without cholesterol (Chol) was tested.

The percentage of encapsulation in all the neutral, anionic and cationic liposomes (Example 5) was 75-90% using a lipid/protein w/w ratio of 50/1 to 300/1, with and without Chol. Table 1 shows the results of an experiment in which the liposomes consisted of the cationic lipid DOTAP. At lipid/protein w/w ratios of  
10 30/1 to 300/1, ~90% antigen encapsulation was achieved, decreasing to 79% and 35% at 10/1 and 3/1 w/w ratios, respectively. The addition of Chol to the formulation did not affect encapsulation at DOTAP/Chol mole ratios of 1/1 and 2/1, with slightly lower encapsulation (80%) at a ratio of 4/1.

HN association with the liposomes upon simple mixing of the soluble  
15 antigen with preformed liposomes was also determined. In such cases, 40-60% of the antigen was associated with the liposomes, regardless of the formulation.

These finding, collectively, indicate very high encapsulation efficiency using a simple and fast (5 min.) procedure in all formulations. Furthermore, even preformed liposomes in aqueous suspension are capable of effectively associating  
20 with the influenza virus surface antigens.

### Immunogenicity of HN-loaded neutral, anionic and cationic liposomes (Lip) administered intranasally

In the first experiment, a comparison was made between neutral (DMPC),  
25 anionic (DMPC/DMPG, 9/1 mole ratio) and cationic (6 formulations) liposomes (Lip) encapsulating the HN antigens to induce local and systemic responses following two i.n. administrations. For all formulations, the lipid/HN w/w ratio was 300/1, and the cationic lipid/Chol or cationic lipid/DOPE mole ratio was 1/1.



Free antigen (F-HN) and F-HN co-administered with cholera toxin (CT, 1 µg) were tested in parallel. The vaccine was given on days 0 and 7, 3 µg/dose (10 µl per nare), and the responses were determined 4-6 weeks after the second vaccine dose.

As shown in Table 2, the free antigen, as well as the neutral and anionic Lip-HN were virtually ineffective mucosal vaccines. In contrast, the cationic Lip-HN, particularly those designated CAT4, CAT5 and CAT6 (the latter comprising of LLC), evoked a robust systemic and mucosal humoral response, with high levels of IgG1, IgG2a and IgA antibodies, namely a mixed Th1+Th2 response. No IgE antibodies were detected. The cationic liposomal vaccines CAT4-CAT6 also induced high levels of IFN $\gamma$  (but not IL-4) in antigen-stimulated spleen cells. The responses produced by CAT6 were even stronger than those induced by F-HN adjuvanted with CT. Based on these findings, only the cationic formulations CAT4, 5 and 6 were further used.

15 **The effect of the lipid/antigen w/w ratio and of liposomal cholesterol (Chol) on the immunogenicity of HN-loaded cationic liposomes**

As mentioned above (Table 1) it was found that: (a) encapsulation efficiency of HN is markedly reduced at lipid/HN w/w ratios below 30/1, and (b) the addition of Chol to the formulations at cationic lipid/Chol mole ratios of 1/1 to 4/1 hardly affects the encapsulation efficiency. Thus the effect of these parameters on the immunogenicity of CAT4, 5 and 6 formulations was further tested. In addition, the immunogenicity of preformed liposomes simply mixed with the soluble antigen was determined. The data shown in Table 3 indicate that all three formulations induce a strong systemic (serum) and local (lung) response, and that lowering the lipid/HN w/w ratio below 100/1 markedly reduces the response. The superiority of CAT6 (the cationic lipid of the present invention) over the other vaccine formulations is again seen as reflected by the high levels of serum and lung IgG2a and IgA antibodies (groups 12-16). Interestingly, simple mixing of soluble antigen

with preformed liposomes generated very potent vaccines (groups 18-20) that are equal to, or even more effective than, liposomes encapsulating the antigen. This suggests that real encapsulation of the antigen may not be necessary for the adjuvanticity of the cationic liposomes. Table 4 shows the results of the effect of Chol in the cationic liposome composition on the immune response. The addition of Chol slightly reduced the systemic HI response to CAT4 at 2/1 and 4/1 mole ratios (groups 4, 5), but not at a 1/1 mole ratio (group 3), and moderately enhances the overall response to CAT5 at all ratios (groups 7-9) and the local (lung) response to CAT6 at a 1/1 ratio (group 11).

10

#### **Effect of the instillation volume on immunogenicity**

After determining the optimal cationic lipid/antigen w/w ratio and the effect of Chol in the liposomal formulation (Tables 3, 4), the influence of the instillation volume. Lip (DOTAP/Chol, 1/1 mole ratio)-HN (CAT5), 3µg/dose, was administered twice i.n. in a total volume of 10, 20, 50 and 100 µl/dose (5, 10, 25 and 50 µl, respectively, per nare) and the response was assessed as described above. As can be seen in Table 5, the highest humoral response was obtained with 20 µl per dose (10 µl/nare). In view of the results presented in Tables 3-5, the following were defined as optimal for i.n. vaccination: (a) lipid/HN w/w ratio of 100-300/1, (b) cationic lipid/Chol mole ratio of 1/1, and (c) instillation volume 10 µl per nare.

20

#### **The effect of adjuvant (CpG-ODN) on the immunogenicity of HN-loaded cationic liposomes**

In the experiment presented in Table 6, an attempt was made to further enhance the response induced by HN-loaded cationic liposomes by the addition of free or liposome-encapsulated CpG-ODN (ODN 1018), shown by present inventors and others (data not shown) to be a potent mucosal adjuvant. In this experiment,

25

free or liposomal (CAT5) HN (2 $\mu$ g/dose) was co-administered i.n. with free or liposomal CpG-ODN (10  $\mu$ g/dose). One group was immunized with HN+CpG-ODN co-encapsulated within the same liposomes. The CpG-ODN was encapsulated in either anionic (DMPC/DMPG, 9/1 mole ratio) or cationic  
5 (DMTAP/Chol, 1/1 mole ratio) liposomes.

The co-administration of free CpG-ODN with F-HN resulted in only a modest enhanced response (group 2). CpG-ODN entrapped in DMPC/DMPG anionic liposomes was 3-10 times (depending on the parameter tested) more efficacious as an adjuvant than free CpG-ODN (group 3) (data not shown). In  
10 comparison, CpG-ODN in DMTAP/Chol cationic liposomes was 7-60 times more potent than free CpG-ODN (group 4), indicating that the cationic liposomes are also a very efficient delivery system for the adjuvant CpG-ODN. However, addition of free or liposomal CpG to HN-loaded DMTAP/Chol liposomes had no beneficial immunostimulatory effect and, in some cases, even decreased the  
15 response (groups 6-8 vs. group 5). The only advantage of co-delivery of CpG-ODN+HN in the same liposomes (group 8) over the other formulations was the higher levels of serum and lung IgG2a antibodies.

These findings suggest that the addition of an exogenous mucosal adjuvant may not be necessary when using potent cationic liposomes, such as CAT4, 5 and 6  
20 (the present invention) for i.n. antigen delivery.

### Induction of cellular responses

Mice were immunized i.n. with various cationic liposomal formulations and the splenocyte cellular responses — cytotoxicity, proliferation and IFN $\gamma$  production — were measured 6 weeks after vaccination. In the experiment shown in Table 7, a  
25 comparison was made between HN-loaded liposomes (groups 3-10) and free antigen (F-HN) admixed with preformed empty liposomes (groups 11-13). We also compared the immunogenicity of CAT5-HN and CAT6-HN (the present invention) prepared at varying lipid/HN w/w ratios (30/1-300/1).

Preferential cytotoxicity against the specific target cells (P815 pulsed with the influenza peptide) was obtained only with CAT6-HN at a lipid/HN w/w ratio of 100/1 (group 8) and with all the three preformed liposomes (CAT4, 5, 6) co-administered with free antigen. The maximum proliferative response was  
5 observed with CAT5-HN at lipid/HN w/w ratios of 50/1 and 30/1 and with CAT6-HN at 300/1, 100/1 and 50/1 ratios. The proliferative and cytotoxic responses elicited by the most efficacious liposomal formulations were 2-3 times greater than those induced by free antigen.

These findings suggest that as compared with the humoral response (Table  
10 3), where the highest levels of all types of antibodies measured were obtained at lipid/HN w/w ratios of 100/1-300/1, lower w/w ratios (e.g. 30/1-100/1) may be optimal for the cellular responses. Moreover, whereas CAT5-HN elicits a strong humoral response, this formulation is a poor inducer of cytotoxic activity, as compared with CAT6-HN. Interestingly, vaccination with mixtures of free antigen  
15 with preformed cationic liposomes (all three formulations) in suspension evokes good cellular responses that are similar in magnitude to those induced by the encapsulated antigen. Thus, simple mixing of free antigen with preformed cationic liposomes may be sufficient to induce both strong humoral (Table 3) and cellular (Table 7) responses. Further studies comparing the efficacy of encapsulated  
20 antigen and antigen-cationic liposome mixtures are warranted.

#### **Effect of the route of vaccine administration on humoral and cellular responses and protective immunity**

In the experiment shown in Table 8, a comparison was made between 1 i.m. dose, 1 or 2 i.n. doses and 2 oral doses of HN-loaded cationic liposomes (CAT4, 5,  
25 6 the latter comprising the LLC of the present invention) with regard to immunogenicity and induction of protective immunity to live virus challenge. Of the various routes, i.n. administration X2 generates the strongest humoral and cellular response and protective immunity. Of the 3 formulations, CAT 6 (the

present invention) induces the highest response, particularly with regards to serum and lung IgG2a and IgA antibodies. Other experiments demonstrated that Cat 4, 5 and 6 (LLC) induce high levels of MHCII, CD40 and B7 molecules in mouse peritoneal macrophages following 48h incubation. CAT6 produces the highest levels. Since these molecules are essential in antigen presentation, these changes in surface molecules of antigen - presenting cells can explain, at least in part, the superior immunostimulatory activity of CAT6.

In Conclusion: Of the 8 different liposomal formulations tested (a neutral, an anionic and six cationic), CAT 6 (the present invention) proves to be the most efficient delivery system for the influenza antigens. This formulation is even more potent than CT, which is regarded as the most powerful mucosal adjuvant, yet toxic and not approved for human use.

## EXAMPLES

### Chemistry

**Example 1:** monospermineceramide-1-O-[N,N-3-aminopropyl-4-(3 aminopropylamino)butyl]carbamoyl-2-hexadecanoylamino-4-octadecene-1,3-diol

- (i) N-palmitoylsphingosine (1.61g, 3mmol) was dissolved in dry THF (100ml) with heating. The clear solution was brought to room temperature and N,N'-disuccinimidyl carbonate (1.92g, 7.5 mmol) was added. DMAP (0.81g, 7.5 mmol) was added with stirring and the reaction further stirred for 16 hours. The solvent was removed under reduced pressure and the residue recrystallized from n-heptane yielding 1.3g (68%) of disuccinimidylceramidyl carbonate as white powder m.p. 73-76°C.
- (ii) Spermine (0.5g, 2.5 mmol) and disuccinimidylceramidyl carbonate (0.39g, 0.5 mmol) were dissolved in dry dichloromethane with stirring and then treated with catalytic amount of DMAP. The solution was stirred at room

temperature for 16 hours, the solvent evaporated and the residue treated with water, filtered and dried in vacuo, giving 0.4g (82%) of crude material which was further purified by column chromatography on Silica gel, using 60:20:20 Butanol: AcOH:H<sub>2</sub>O eluent.

- 5 (iii) Methylation of the compound obtained in (ii) with DMS or CH<sub>3</sub>I.

#### Example 2: Preparation of Liposomes

Liposomes were prepared by dissolving the appropriate cationic lipid (DOTAP-1,2-dioleoyl-3-trimethylammonium-propane, DC-CHOL-3β[N-(N',N'-  
10 dimethyl-aminoethane)carbonyl]cholesterol or the product of Example 1) or cationic lipid/neutral co-lipid (DOPC-1,2-dioleoyl-*sn*-glycero-3 phosphatidylcholine, DOPE-dioleoylphosphatidylethanol-amine or cholesterol) mixture (1/1 mole ratio) in tert-butanol. The mixture was centrifuged under vacuum for 3 hours in an Automatic Environmental Speedvac, Model AE2010 (Savant Instruments Inc,  
15 Holbrook, NY) such that all the tert-butanol had evaporated. The powder thus formed was hydrated with 20mM hepes buffer (pH 7.4) to produce a heterogeneous vesicle (HV) dispersion exhibiting a total lipid concentration of 31mM. If required, these HVs, exhibiting ~500–1500 nm diameter, could be downsized and converted to large unilamellar vesicles (LUVs), exhibiting ~100 nm diameter, by the use of a  
20 'Liposofast' extrusion system (MacDonald et al. 1991) as previously described (Meidan et al. 2000). In all cases, the prepared liposomes were stored at 4°C. Just before their addition to cells, small volumes of liposome dispersion were diluted with pre-calculated amounts of 20mM Hepes buffer (pH 7.4) in order to produce aliquots containing the appropriate amount of cationic lipid.

#### 25 Example 3: Preparation of Lipoplexes containing oligonucleotides

For the preparation of the lipoplexes, the diluted liposome dispersions (either HV or LUV) obtained in Example 2 were added to solutions comprising an 18-mer antisense phosphorothioate oligonucleotide (S-ODN), appropriately diluted in 20mM Hepes buffer at pH 7.4, in glass vials at the appropriate

L<sup>+</sup>/ODNP<sup>-</sup> mole ratio. It should be noted that each cationic lipid molecule in the compound formed in Example 1 exhibits an equivalent of two positive charges and this was taken into consideration for calculating L<sup>+</sup>. In all cases, lipoplexes were formed by allowing the mixture to stand for 20 to 40 min with occasional mild  
5 vortexing (Meidan et al.).

#### Example 4: Lipofection

150µl Aliquots of culture medium (incorporating 10% v/v fetal calf serum),  
10 each containing  $4 \times 10^3$  cells, were deposited into the wells of a flat bottom 96 well plate (Corning Inc, NY). A 50µl volume of the appropriate treatment solution was then added to each seeded well. The treatment solutions contained S-ODN (generally 0.005 nmol unless otherwise stated), either free or as freshly prepared lipoplexes; or alternatively control liposome solutions containing equivalent  
15 amounts of cationic lipid. In all cases, the cells were incubated for 96 h at 37°C, 90% relative humidity and 5% CO<sub>2</sub>. Each specific treatment solution was added to three different wells and the entire experiment was performed in triplicate, each time using a different batch of cultivated cells. *i.e.* each treatment solution was added to 9 wells together.

#### 20 Example 5: Encapsulation of antigens in liposomes

The HN antigens {influenza A/New Caledonia/20/99-like (H1N1)} were encapsulated in large (mean diameter 0.5-1 µm) heterogeneous vesicles (HV) consisting of either: (a) dimyristoyl-phosphatidylcholine (DMPC, neutral liposomes); (b) DMPC and dimyristoyl-phosphatidylglycerol (DMPG) (both from  
25 Lipoid GmbH, Ludwigshafen, Germany) at a DMPC:DMPG mole ratio of 9:1 (anionic liposomes); or (c) cationic (CAT) lipids, alone and with cholesterol (Chol) at 1:1 to 4:1 mole ratio, respectively, or with the neutral lipid dioleoyl-phosphatidylethanolamine (DOPE) at a 1:1 mole ratio (cationic liposomes). The following commercial cationic lipids (a-e) were used:

30 (a) Dimethylaminoethane-carbamol-cholesterol (DC-Chol:DOPE) (CAT1),

- (b) Distearoyl-trimethylammonium-propane-cholesterol (DSTAP) (CAT2),
- (c) Dimethyldioctadecylammonium-bromide-cholesterol (DDAB) (CAT3),
- (d) Dioleoyl-trimethylammonium-propane-cholesterol (DOTAP) (CAT4),
- (e) Dimyristoyl-trimethylammonium-propane-cholesterol (DMTAP) (CAT5) and
- (f) The lipid-like cationic (LLC) vehicle of the present invention-cholesterol (CAT6) as synthesized by the inventors.

For all formulations, the following procedure was used. Lipids (10-30 mg) were dissolved in 1ml tertiary butanol, then sterilized by filtration (GF92, Glasforser, Vorfilter no. 421051, Schleicher & Schuell, Dassel, Germany). The sterile lipid solution was frozen at -70°C, then lyophilized for 24 h to complete dryness. The dried lipids could be stored at 4°C for >1 year without significant (<10%) lipid degradation or loss or encapsulation capability. Upon need, the lipid powder was hydrated with the antigen solution (in saline) at a lipid:antigen w/w ratio of 3:1 to 300:1. The antigen solution was added stepwise in increments of 30-50 µl and vortexed vigorously after each addition, up to a final volume of 0.5-1 ml. In some experiments, the antigen solution was mixed, using vortex, with preformed empty liposomes suspended in saline or PBS.

To determine encapsulation efficiency, liposomes were centrifuged under conditions where  $\geq 90\%$  of the liposomes precipitate while  $\geq 80\%$  of the non-encapsulated antigen remains in the supernatant. Non-cationic liposomes containing the influenza HN antigens were centrifuged at 4°C for 30 min at 14,000 rpm and the amount of non-encapsulated protein was determined by the modified Lowry technique. For the cationic liposomes, a sample (0.5 ml) was loaded very carefully using a gel loading tip (100-150 µl at a time) over 0.5 ml of D<sub>2</sub>O, then centrifuged for 1 h at 30°C at 45,000 rpm. This results in the free, non-encapsulated HN precipitating into a pellet at the bottom of the eppendorf tube, while the liposomal HN and a few empty liposomes float at the top of, or in the supernatant. To determine encapsulation, the entire supernatant was collected



with a Pasteur pipette. The liposomes and the pellet were then dissolved with 200  $\mu$ l of warm 10% Triton X-100 added to each fraction. The Lowry method (modified to 20% SDS procedure) was used to determine protein concentration in each fraction.

5

#### Example 6: Immunization

Free (F-HN) and liposomal (Lip-HN) vaccines, 1-4  $\mu$ g/dose, were administered either once intramuscularly (i.m., in 30  $\mu$ l), once or twice intranasally (i.n., in 5-50  $\mu$ l per nare), or twice orally (in 50  $\mu$ l), spaced 1 week apart. In all  
10 cases, mice were lightly anesthetized with 0.1 ml of 4% chloral hydrate in PBS given intraperitoneally. For oral administration mice were treated with an antacid 30 min prior to vaccination. Cholera toxin (CT, Sigma, USA), 1  $\mu$ g/dose, was used in all experiments as a standard mucosal adjuvant for comparison. In one  
15 experiment, CpG-ODN (ODN 1018 generously provided by Dr. E. Raz, University of California, San Diego, CA, USA), 10  $\mu$ g/dose, was used as an adjuvant.

#### Example 7: Assessment of humoral responses

Sera, lung homogenates and nasal washes, prepared as described previously, were tested, individually or pooled, 4-6 weeks post-vaccination, starting at 1/10 or  
20 1/20 sample dilution. Hemagglutination inhibiting antibodies were determined by the standard hemagglutination inhibition (HI) assay. Mice with HI titer  $\geq$ 40 (considered a protective titer in humans) were defined as seroconverted. Antigen-specific IgG1, IgG2a, IgA and IgE levels were measured by ELISA. The highest sample dilution yielding absorbance of 0.2 OD above the control (antigen +  
25 normal mouse serum, OD <0.1) was considered the ELISA Ab titer.

#### Example 8: Assessment of cellular responses

Splenocytes obtained at 4-6 weeks after vaccination were tested for proliferative response, IFN $\gamma$  and IL-4 production, and cytotoxic activity, following

*in vitro* stimulation with the antigen. Cultures were carried out at 37°C in enriched RPMI 1640 or DMEM medium supplemented with 5% (for proliferation, cytokines) or 10% (for cytotoxicity) FCS, with (for cytotoxicity) or without  $5 \times 10^{-5}$  M 2-mercaptoethanol. Cell cultures were performed as follows, (i) Proliferation:  $0.5 \times 10^6$  cells per well were incubated in U-shaped 96-well plates, in triplicate, with or without the antigen (0.5-5 µg per well), in a final volume of 0.2 ml. After 72-96h. cultures were pulsed with 1 µCi  $^3$ H-thymidine for 16 h. Results are expressed in  $\Delta$ cpm = (mean counts per minute of cells cultured with antigen) - (mean counts per minute of cells cultured without antigen). (ii) Cytokines:  $2.5 \times 10^6$  to  $5 \times 10^6$  cells per well were incubated in 24-well plates, in duplicate, with or without the antigen (5-10 µg per well), in a final volume of 1 ml. Supernatants were collected after 48-72 h and tested by ELISA for murine IFN $\gamma$  and IL-4 using the Opt ELA Set (Pharmingen, USA). (iii) Cytotoxicity: Responding splenocytes ( $2.5 \times 10^6$ ) were incubated as in (ii) for 7 days together with an equal number of stimulating BALB/c splenocytes that had been infected with the X/127(H1N1) influenza virus (see below). For infection, the splenocytes were incubated, with occasional stirring, for 3 h at 37°C in RPMI 1640 medium (without FCS) with 150 hemagglutination units/ $1 \times 10^6$  splenocytes of the virus, followed by washing. Subsequently, the primed effector cells were restimulated for 5 days with infected, irradiated (3,000 rad) splenocytes at an effector/stimulator cell ratio of 1/4 in the presence of 10 IU/ml of rhIL-2. Cytotoxicity was measured using the standard 4 h  $^{51}$ Cr release assay at effector/target cell ratios of 20/1 and 100/1. The labeled target cells used were unmodified P815 and P815 pulsed for 90 min at 37°C with the HA2 189-199 peptide (IYSTVASSLVL, 20 µg/ $1 \times 10^6$  cells).

25

#### Example 9: Determination of protective immunity

Mice were anesthetized and 25 µl of live virus suspension per nostril was administered, using the reassortant virus X-127 (A/Beijing/262/95 (H1N1) x X-31 (A/Hong Kong/1/68 x A/PR/8/34), which is infectious to mice and cross-reactive

with A/New Caledonia,  $10^7$  EID 50 (egg-infectious dose 50%). The lungs were removed on day 4, washed thrice in cold PBS, and homogenized in PBS (1.5 ml per lungs per mouse, referred to as 1/10 dilution). Hemogenates of each group were pooled and centrifuged at 2000 rpm for 30 min at 4°C and the supernatants collected. Serial 10-fold dilutions were performed and 0.2 ml of each dilution was injected, in duplicate, into the allantoic sac of 11-day-old embryonated chicken eggs. After 48 h at 37°C and 16 h at 4°C, 0.1 ml of allantoic fluid was removed and checked for viral presence by hemagglutination (30 min at room temperature) with chicken erythrocytes (0.5 wt.%, 0.1 ml). The lung virus titer is determined as the highest dilution of lung homogenate producing virus in the allantoic fluid (positive hemagglutination).

#### Statistical analysis

Differences in HI antibody titer between groups were analyzed using a two-tailed Student's *t*-test. *P*-values <0.05 were considered significant.

## MATERIALS AND METHODS

### *Materials*

**S-ODN:** Phosphorothioate antisense 18-mer-G3139 [S-d-5'-(TCT CCC AGC GTG CGC CAT)] was obtained from Genta Inc. Lexington, MA. Its purity was assessed by HPLC (Merck Hitachi D-7000) incorporating an anion exchange column. A gradient of ammonium acetate in 50% isopropanol in a pH 8 buffer was employed. For each ODN, a single sharp peak was obtained, thus confirming that our supplies of both G3139 and G3622 were indeed pure. The ODNs were dissolved in 20 mM Hepes buffer (pH 7.4) and ODN concentrations were determined by optical density measurements (Shimadzu UV-24101PC, Duisburg, Germany) made at 254 nm (Brown T, Brown DJS, 1991. Modern machine-aided methods of oligonucleotide synthesis, In: Eckstein F editor. Oligonucleotides and Analogues, A Practical Approach. New York: Oxford University Press, pp. 20). By multiplying the derived

ODN concentration values by 17, it was possible to obtain the corresponding phosphate anion (ODNP<sup>-</sup>) concentration. The ODN phosphate concentration was confirmed by a direct organic phosphorus determination.

#### Cell culture

5 MCF-7 cells were cultivated in DMEM supplemented with 2mM L-glutamine, 100 IU/ml penicillin-streptomycin, 4.5g/L D-glucose, 1 mM sodium pyruvate and 10% v/v fetal calf serum. For the lipoplex studies, the MCF-7 cells were cultivated as above with the exception that no pyruvate was incorporated. The cells were grown to ~90% confluency in 75cm<sup>2</sup> flasks. All the cell culture  
10 medium components were acquired from Biological Industries (Kibbutz Beit Haemek, Israel).

#### Western Blot

Cell extracts were obtained by lysing the cells in protein lysis buffer (Tris-HCl pH 6.8, 2.24% glycerol, 6% SDS, 0.02% bromophenol blue, 10%  
15  $\beta$ -mercaptoethanol) for 10 min at 100°C and subsequent centrifugation. The extracts were subjected to polyacrylamide gel electrophoresis (PAGE-SDS). Bradford dye assay (Bio Rad Laboratories, Hercules, CA) indicated that each lane contained 50 $\mu$ g of protein. Anti human bcl-2 mAb (100) (Santa Cruz Biotechnolgy, Inc., Santa Cruz, CA) was used. Detection was performed by  
20 employing horse radish peroxidase conjugated to goat anti-mouse IgG (H+L) (Jackson Immuno Research Lab. Inc., West Grove, PA), followed by ECL (Amersham, Pharmacia Biotech, Uppsala, Sweden). Antibodies were stripped from the membrane by 1% SDS in phosphate-buffered saline. The detection of actin levels by the use of anti-human actin mAb (Roche Molecular Biochemicals,  
25 Annheim, Germany) served as an internal control.

#### **Luciferase assay**

Luciferase activity was assayed on cell extracts using Bright-Glo™ Luciferase Assay system (Promega, Madison WI). Briefly, the cell were washed 2 times with cold PBS x 1 and lysed for 10 min with Cell Culture Lysis Buffer (Promega). Then, cells were harvested and centrifuged for 5 min to remove cell debris. The amount of enzyme was measured on Turner luminometer (Promega) quantified in supernatants and was quantified by generating calibration curve using recombinant enzyme QuantiLum® (Promega, Madison WI). The amount of luciferase was normalized to the amount of total protein in the lysate, which was  
10 determined by Lowry method.

#### **Mice**

Specific pathogen-free (SPF) female BALB/c mice, 6-8 weeks old, were used (5-10 per group). Animals were maintained under SPF conditions.

#### **Influenza antigens**

15 A monovalent subunit antigen preparation derived from influenza A/New Caledonia/20/99-like (H1N1) strain was generously provided by Drs. Gluck and Zurbriggen, Berna Biotech, Bern, Switzerland. This preparation (designated herein HN) comprised of 80-90% hemagglutinin, 5-10 wt.% neuraminidase and trace amounts of NP and M1 proteins. A whole-inactivated virus was used in some  
20 experiments for in vitro stimulation.

The invention will now be defined by the appended claims, the contents of which are to be read as included within the disclosure of the specification.

**ADDITIONAL EXAMPLE: Characterization of the cationic lipid of the invention**

In the following the cationic lipid of the invention is referred to by the terms:

CCS#5A - designating the acetate salt of the cationic lipid; or

CCS#5C - designating the chloride salt of the cationic lipid

TNBS assay was used to determine primary amine concentration in the cationic lipid of the invention. Accordingly, the tested compound (100 ml) was added to bicarbonate solution (500µl, pH 8.4) followed by the addition of TNBS (1200nmol, 20µl). The mixture was incubated for 30 min. After incubation, acetic acid was added (20 µl). Red sediments were spun down and dissolved in acetonitril. Mass spectra of the product was obtained.

Figures 11A-11C presents Mass Spectra of the product of reacting TNBS with the acetate salt of cationic lipid of the invention (CCS#5A). The product is referred to by the name CCS-TNP. According to the Mass Spectra of TNBS derivatized Ceramide-spermine, there is only one primary amine in CCS (peak 978.9), i.e. if there were two amine groups in the cationic lipid of the invention, they would have both reacted with TNBS to give CS-2TNB. To verify that a second amine did not react with TNBS as a result of steric hindrance, spermine alone was reacted with TNBS. Fig. 1B shows that spermine reacted with TNBS at both free amines.

In view of the above results and Mass Spectra of the cationic lipid of the invention it was concluded that the cationic lipid of the invention has only one free primary amine and has the chemical structure shown in Fig. 11C.

Physical characterization of the cationic lipid of the invention was also performed.

The following data presents physical characterization of the cationic lipid of the invention, as an acetate (Acetate) or chloride salt (Chloride), as the salt or in combination with DOPE (ratio of CAT/DOPE 2:1) or in combination with cholesterol (ratio CAT/Chol 2:1).

### Critical micelle concentration (CMC)

CMC was measured by changes in DPH fluorescence upon aggregation.

Acetate=Chloride 5.00E-06M

### Liposome size, nm

Size (mass-weight) was measured with non-invasive back scattering ALV instrument (ALV GmbH)

Chloride 25  
Chloride/DOPE 2:1 3594  
Acetate 6  
Acetate/DOPE 2:1 498

### Electrostatics of liposomes (different salt and different counter-ion)

pKa determined from dissociation curve of hydroxycoumarin (HC) or hydroxycoumarin phosphatidyl ethanolamine (HC-PE) (in parenthesis). Zeta-potential determined in 10 mM NaCl with Zetasizer (Malvern instruments)

	pKa	Surface potential, mV	Surface pH	Zeta-potential
DOPE/DOPC 1:1	10.7 (8.95)	0	7.4	0
Acetate	(4.75)	(245.8)	(11.5)	40
Acetate/DOPE 2:1	7.54 (4.93)	184.9 (235.2)	10.56 (11.32)	43.4
Chloride	6.56 (5.61)	242.3 (195.4)	11.54 (10.64)	46.5
Chloride/DOPE 2:1	7.41 (5.94)	192.5 (176.15)	10.69 (10.31)	86.8
Base	7.23	203.06	10.87	34
Base/DOPE 2:1	8.65	119.97	9.45	
Chloride/Chol 2:1				29.5/2.3

The titration of different the liposomes formed from the cationic lipid of the invention [the acetate salt of the cationic lipid of the invention ("Acetate"), the combined acetate salt and DOPE (ratio 2:1, "Acetate/DOPE"), the chloride salt ("Chloride") or the combined chloride salt and DOPE (ratio 2:1, "Chloride")] is presented in Figure 12.

In addition, hydration of different liposomes before and after the addition of plasmid DNA was determined. Hydration was monitored by changes of Laurdan excitation general polarization according to which the higher the value obtained, the lower is the hydration. The results obtained are presented in Fig. 13.

The effect of storage of the cationic lipid of the invention was also evaluated. Figures 14A to 14E present different follow-up assays of storage of the cationic lipid in HEPES, at 4°C, alone or combined with DOPE (ratio 2:1).

The conclusions which may be drawn from these bar diagrams is that:

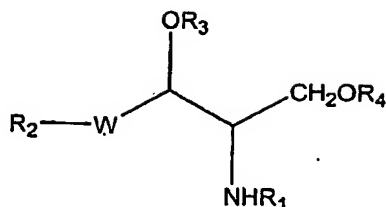
- No substantial degradation of lipid occurred upon storage in hydrated form in HEPES buffer (pH 7.4) at 4°C (no change in TNBS-primary amine) up to 23 days.
- No substantial change in surface and zeta-potential occurred;
- Aggregation upon storage that leads to decrease in transfection activity, was easily overcome by sonication of the liposomes for 10 seconds.

Finally, light microscopy and Cryo-TEM images were obtained and are presented in Figures 15A-15C (light microscope images) and in Figures 16A-16D (Cryo-TEM).



CLAIMS:

1. A compound of formula (I):



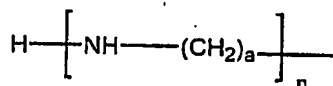
wherein

- 5  $\text{R}_1$  is selected from hydrogen, branched or normal alkyl, aryl, lower alkyl amine wherein said amine may be substituted, or  $\text{C}(\text{O})\text{R}_5$ ;

$\text{W}$  is  $-\text{CH}=\text{CH}-$ ,  $-\text{CH}_2-\text{CH}(\text{OH})-$  or  $-\text{CH}_2-\text{CH}_2-$ ,

$\text{R}_2$  and  $\text{R}_5$  are independently selected from a saturated or unsaturated normal or branched  $\text{C}_{10}-\text{C}_{24}$  alkyl, alkenyl or polyenyl groups;

- 10  $\text{R}_3$  and  $\text{R}_4$  are independently selected from hydrogen, a group  $-\text{C}(\text{O})-\text{NR}_6\text{X}-\text{Z}$ ,  $\text{Z}$  being a same or different for  $\text{R}_3$  and  $\text{R}_4$ ; or  $\text{R}_3$  and  $\text{R}_4$  form together with the oxygen atoms to which they are bound a heterocyclic group comprising  $-\text{C}(\text{O})-\text{NH}-\text{Z}-\text{NH}-\text{C}(\text{O})-$ ;  $\text{R}_6$  is hydrogen or

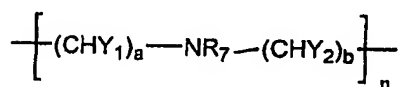


- 15 wherein  $a$  represents an integer from 1 to 4;  $n$  represents an integer from 1 to 6;

$\text{Z}$  being a branched or linear polyalkylamine chain wherein said polyalkylamine chain may comprise quaternary ammonium groups;

with the proviso that  $\text{R}_3$  and  $\text{R}_4$  are not simultaneously hydrogen.

2. The compound of Claim 1, wherein said polyalkylamine chain has  
20 the general formula (II):

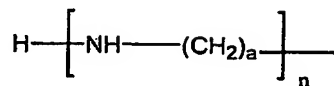


wherein

a and b are independently selected from 1, 2, 3 or 4;

n represents an integer from 1 to 6;

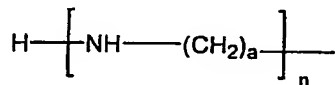
R<sub>7</sub> is selected from hydrogen or



5

where a and n have the meaning as above; and Y<sub>1</sub> and Y<sub>2</sub> are independently selected from hydrogen and alkyl.

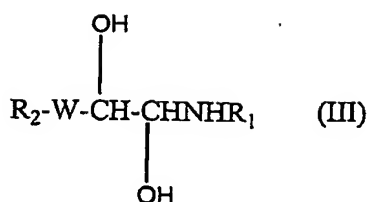
3. A compound according to claim 2 wherein R<sub>1</sub> is a C(O)R<sub>5</sub> group, W is -CH=CH-, R<sub>2</sub> and R<sub>5</sub> are independently selected from a saturated or unsaturated  
10 linear or branched C<sub>12</sub>-C<sub>18</sub> alkyl or alkenyl groups; R<sub>3</sub> and R<sub>4</sub> are independently selected from hydrogen and -C(O)-NR<sub>6</sub>X-Z, R<sub>6</sub> is hydrogen or



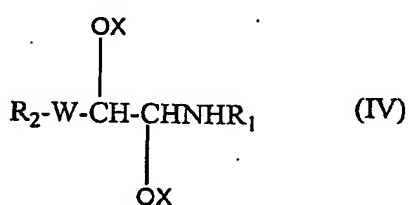
and Z is a polyalkylamine chain.

4. A compound according to claim 2 wherein wherein R<sub>1</sub> is a C(O)R<sub>5</sub>  
15 group, W is -CH=CH-, R<sub>2</sub> and R<sub>5</sub> are independently selected from a saturated or unsaturated linear or branched C<sub>12</sub>-C<sub>18</sub> alkyl or alkenyl groups; R<sub>3</sub> and R<sub>4</sub> form together with the hydrogen atoms to which they are bonded a heterocyclic group comprising -C(O)-NH-Z-NH-C(O)-, Z being a branched or linear polyalkyl amine chain wherein said polyalkylamine chain may comprise quaternary ammonium  
20 groups.

5. A process for the preparation of a compound of formula (I) comprising the steps of reacting a compound of formula (III)



wherein R<sub>1</sub>, R<sub>2</sub> and W have the meaning as defined above;  
with an activating compound selected from N,N'-disuccinimidylxycarbonate, di-  
or tri-phosgene or an imidazole residue to form a compound of formula (IV)



wherein X is a succinimidylxycarbonyl, a chloroformate or an imidazole residue;  
said compound of formula (IV) being further reacted with one or two equivalents  
of branched or linear polyalkylamine to form a compound of formula (I).

6. A method for introducing biologically active compounds into  
eukaryotic cells comprising forming liposomes from the compound of formula (I),  
contacting said liposomes with a biologically active compound to form a complex  
and administering said complex.

7. A complex comprising liposomes formed from the compound of  
formula (I) and a biologically active compound.

8. A complex according to claim 7 wherein said biological active  
compounds are selected from the group comprising of genes, polynucleotides,  
oligonucleotides, proteins, antigens, peptides and drugs.

9. A pharmaceutical composition comprising a complex as claimed in  
claim 7 together with suitable excipients.

10. A pharmaceutical composition according to claim 9, which is  
administered intravenously, subcutaneously, topically, intranasally, orally, ocularly,  
intramuscularly.

11. A method of treatment of cancer in a subject comprising the administration a pharmaceutical composition as claimed in claim 9 to said subject.

12. A method according to claim 11, wherein said cancer is breast cancer.

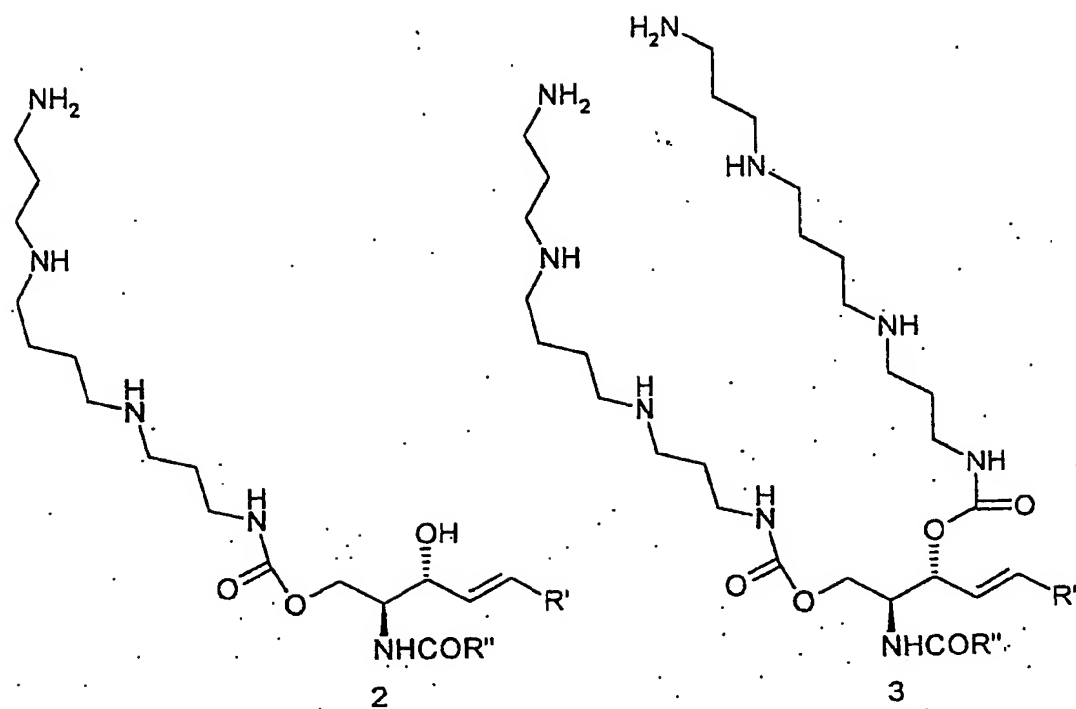
5 13. A method of vaccination of a subject in need thereof comprising the administration of a composition as claimed in claim 9.

14. A method according to claim 13 wherein said vaccination is done mucosally, preferably intranasally.

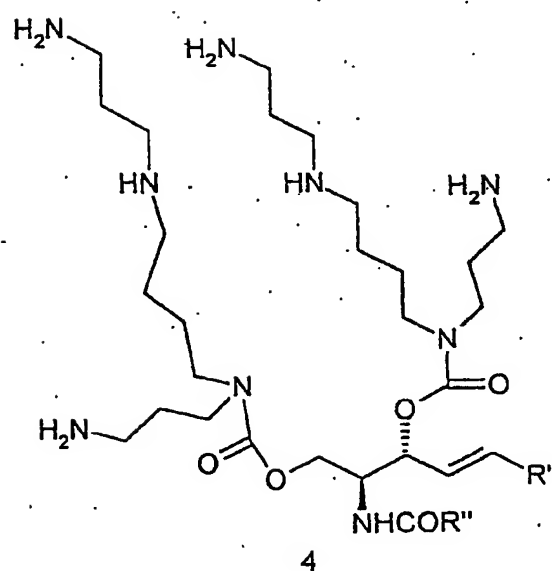
15. A method of transfection of a gene into cells comprising contacting a  
10 complex as claimed in claim 7 with cells.

**Abstract**

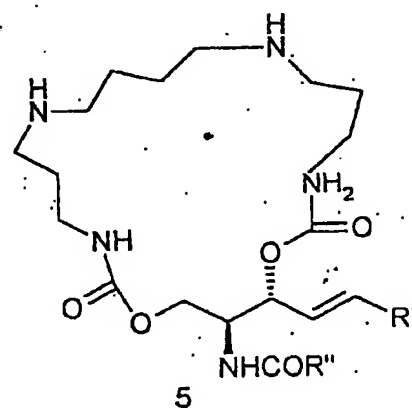
The present invention concerns compounds of formula (I) and their use as lipid-like vehicles for delivering active biological moieties into cells for therapy, immunization and gene transfer.



"linear" ceramide-Sprm conjugates



"branched" ceramide-Sprm conjugate



"cyclic" ceramide-Sprm conjugate

Fig. 1

batch5 #16-44 RT: 0.42-0.92 AV: 29 NL: 2.95E6  
T: + p ms [ 200.00-1200.00]

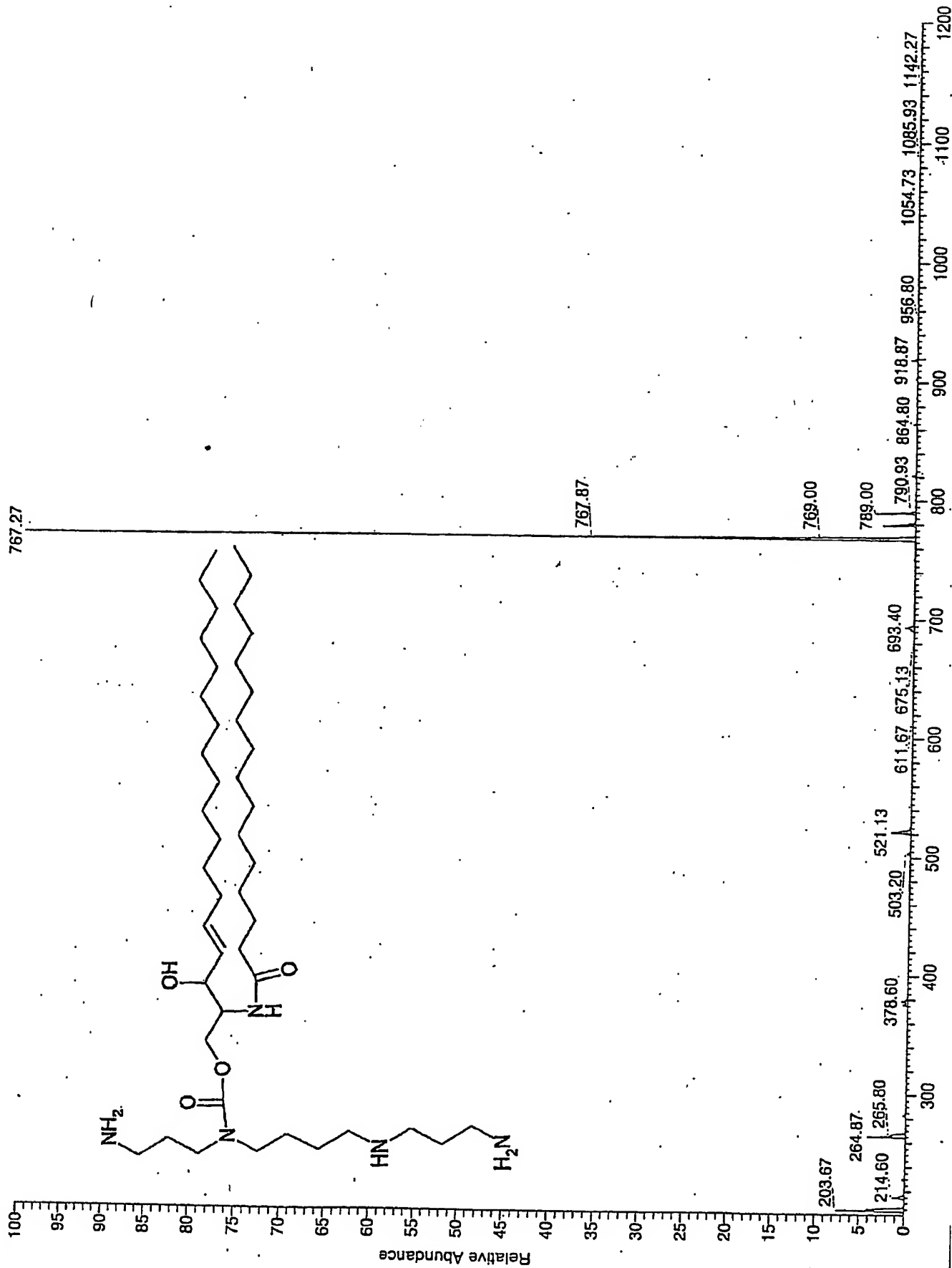


Fig. 2

Fig. 6A

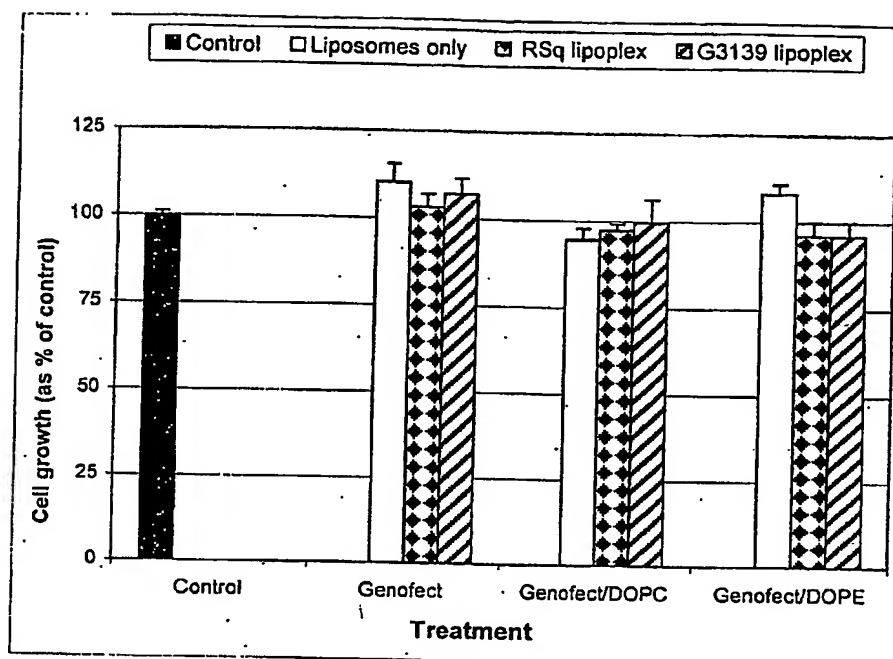


Fig. 6B

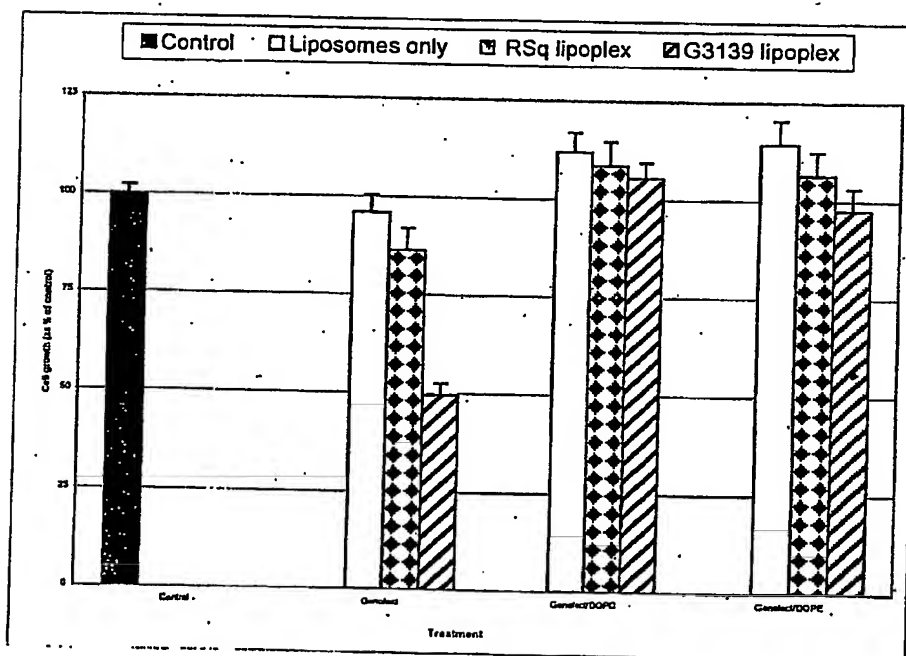
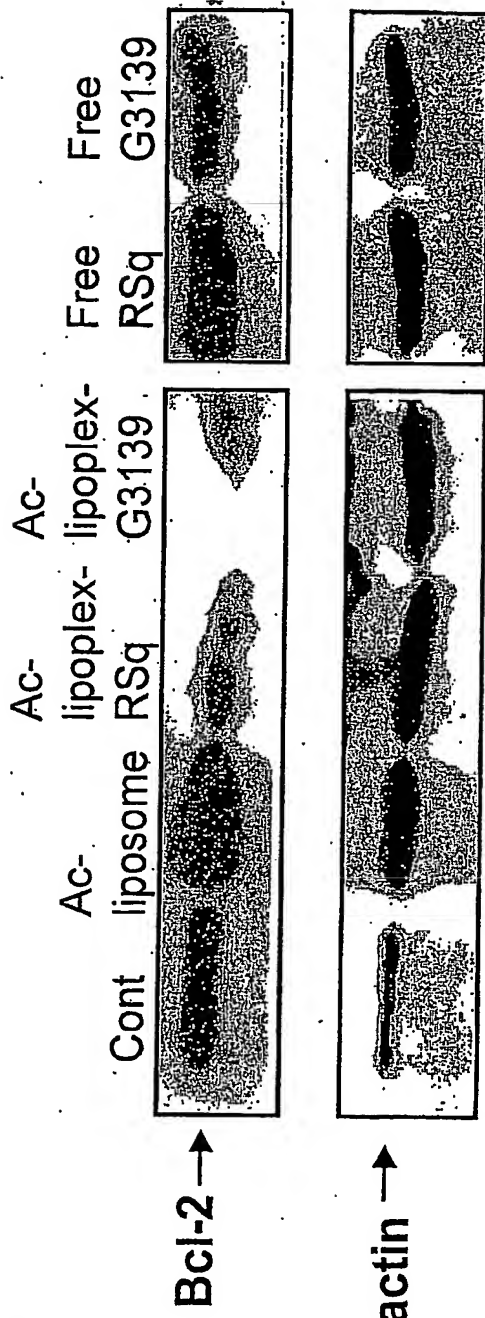


Fig. 3



**A**



**B**

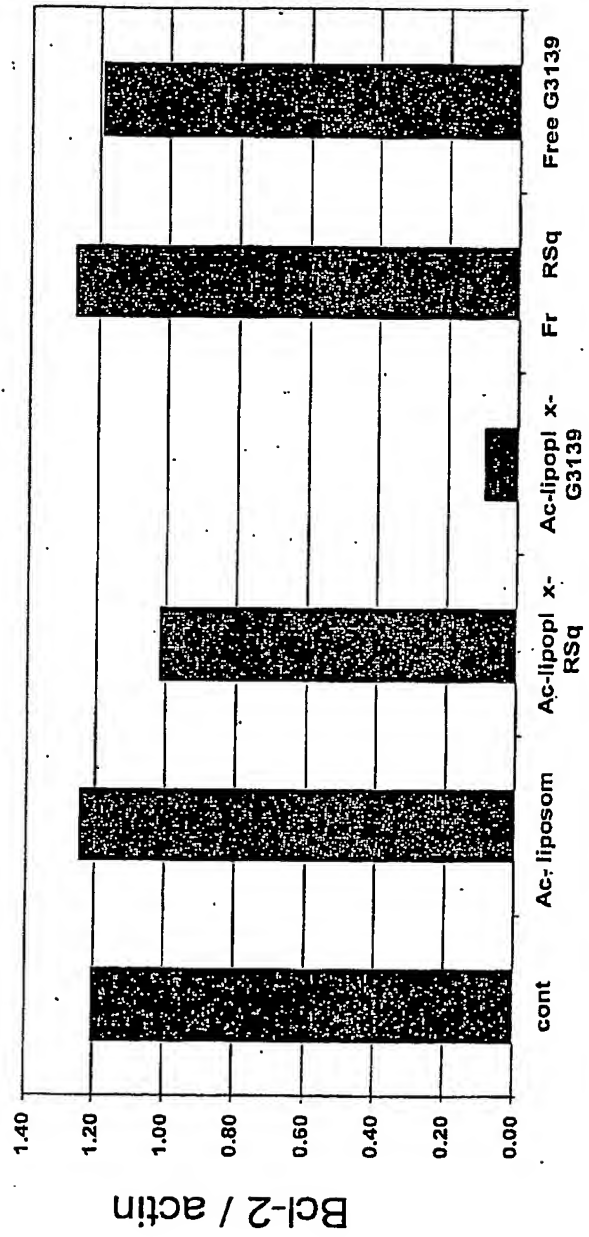


Fig. 4

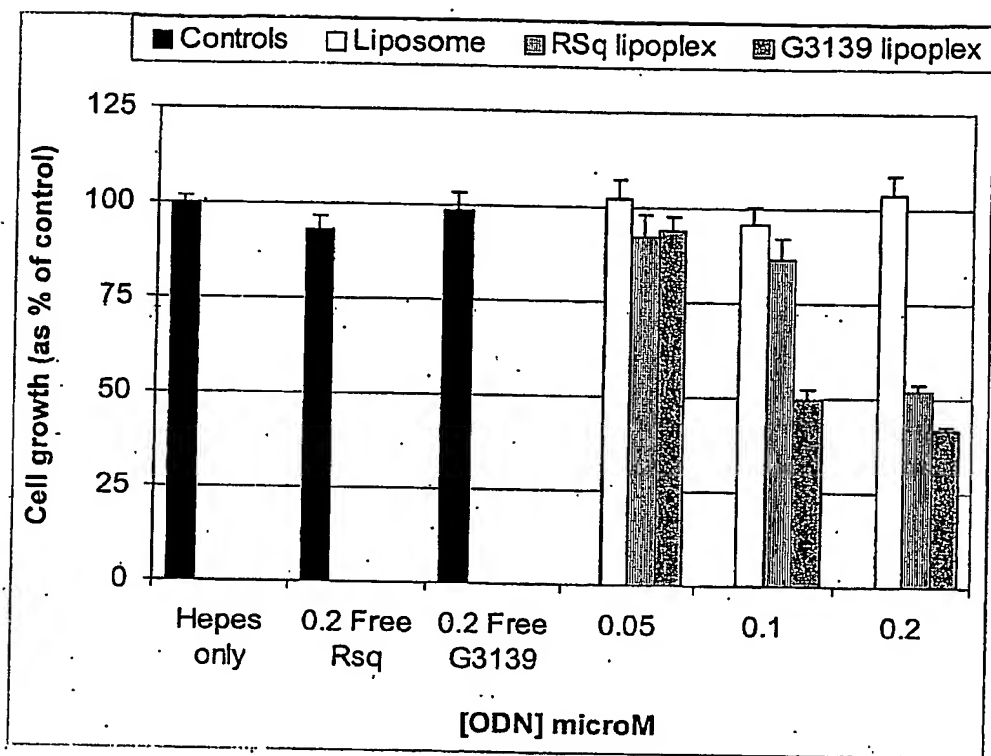


Fig. 5

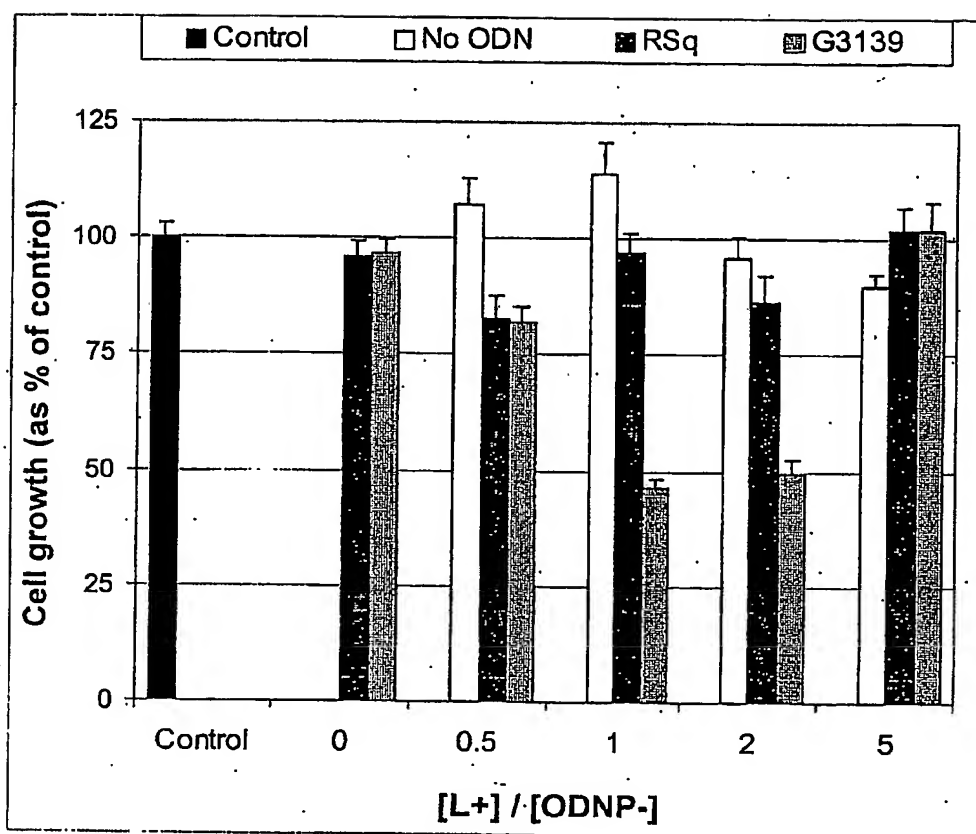


Fig. 6

# Toxicity of B5C on NIH 3T3

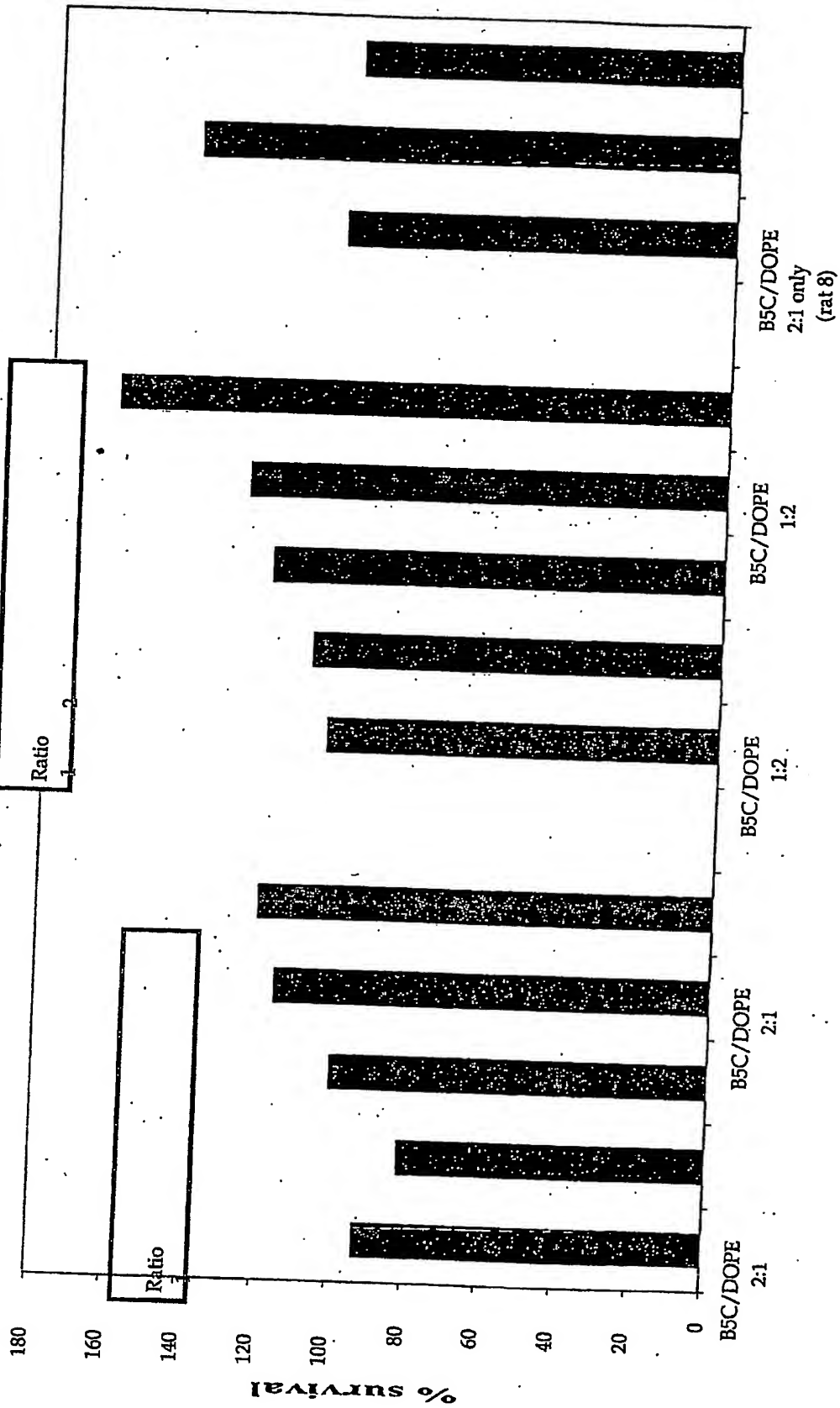


Fig. 7

Luciferase transfection of NIH 3T3 with B5C-effect of DOPE

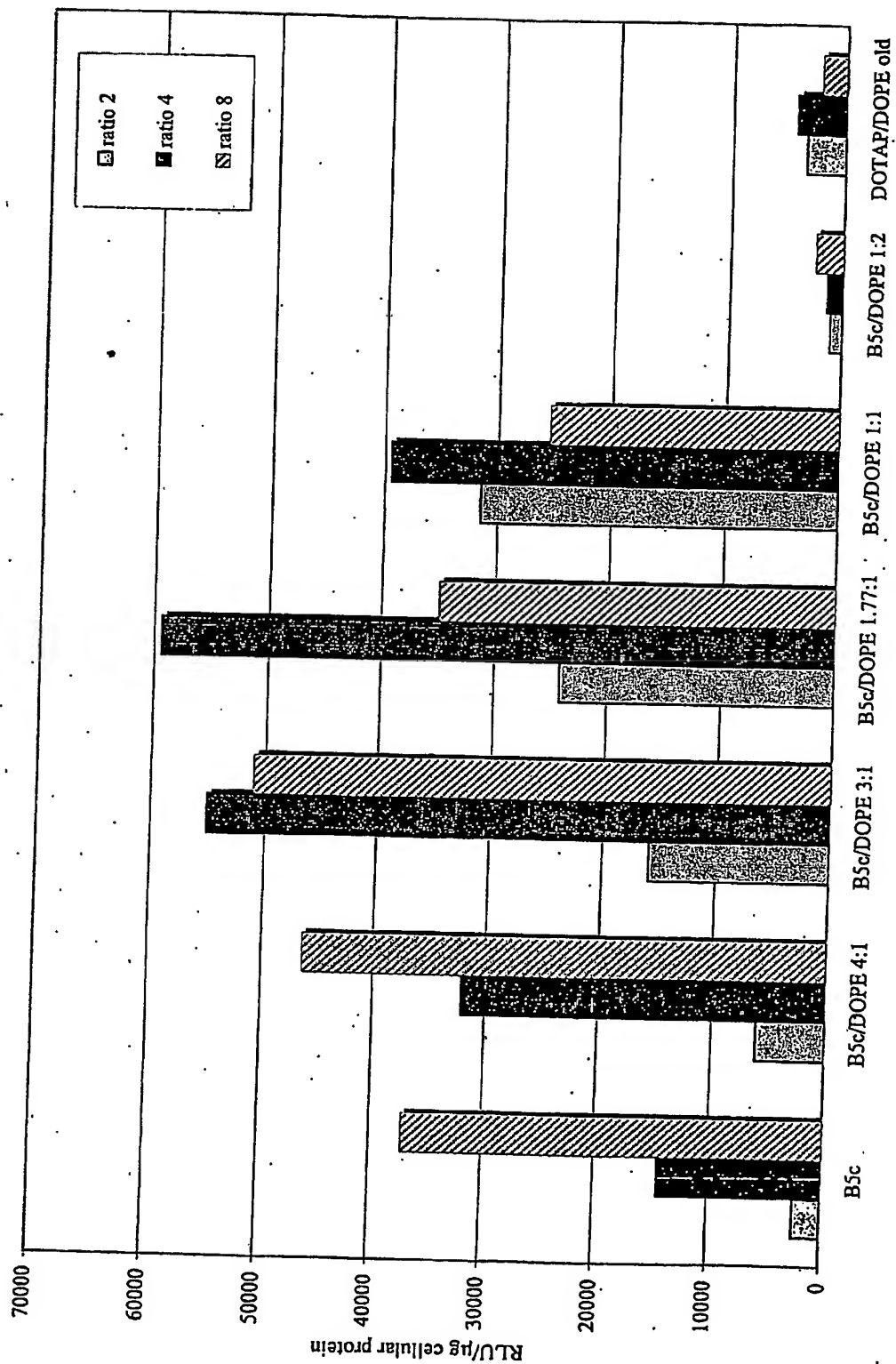


Fig. 8

Transfection of C-26 cells with  
 luciferase: comparison with  
 FuGENE transfection reagent

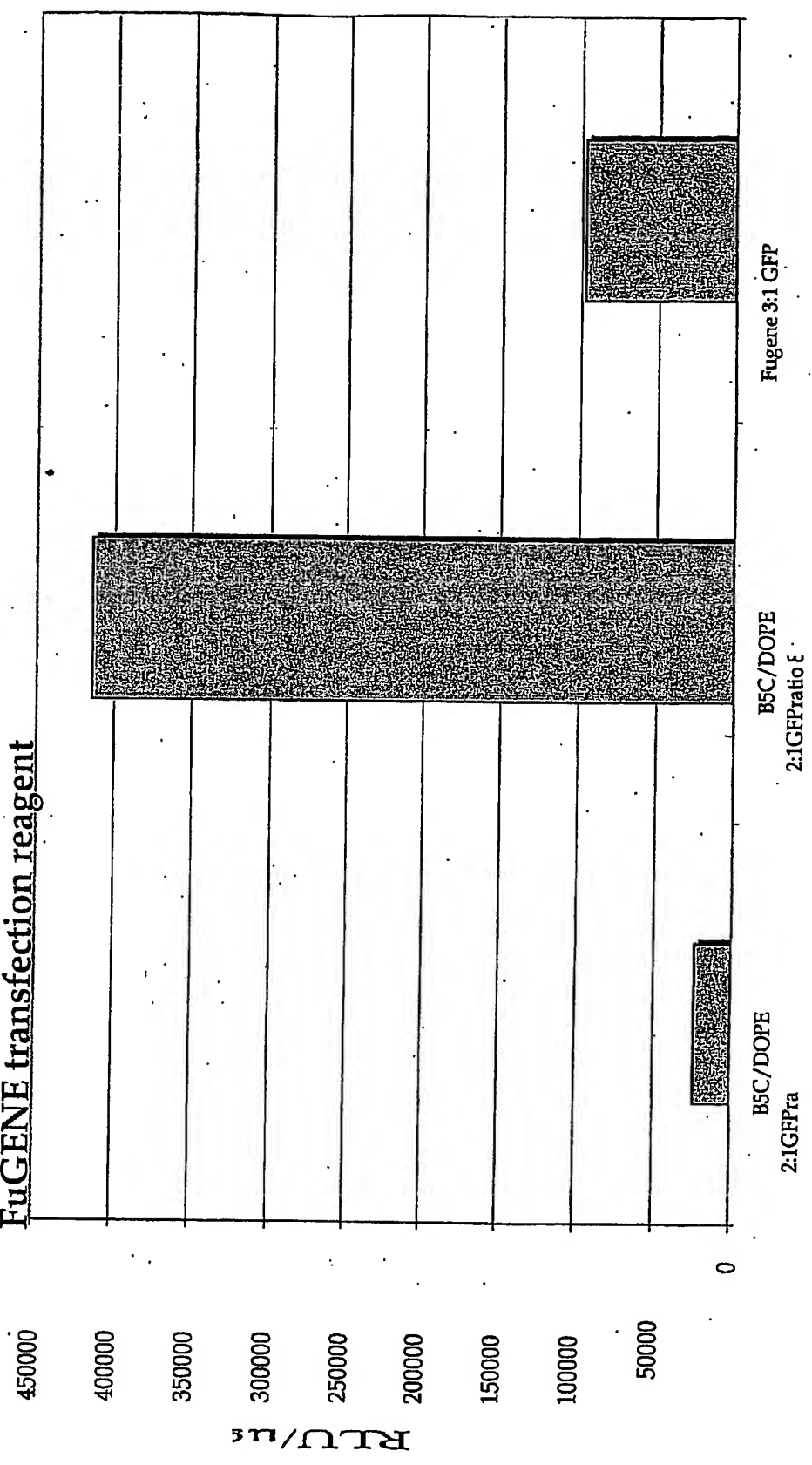
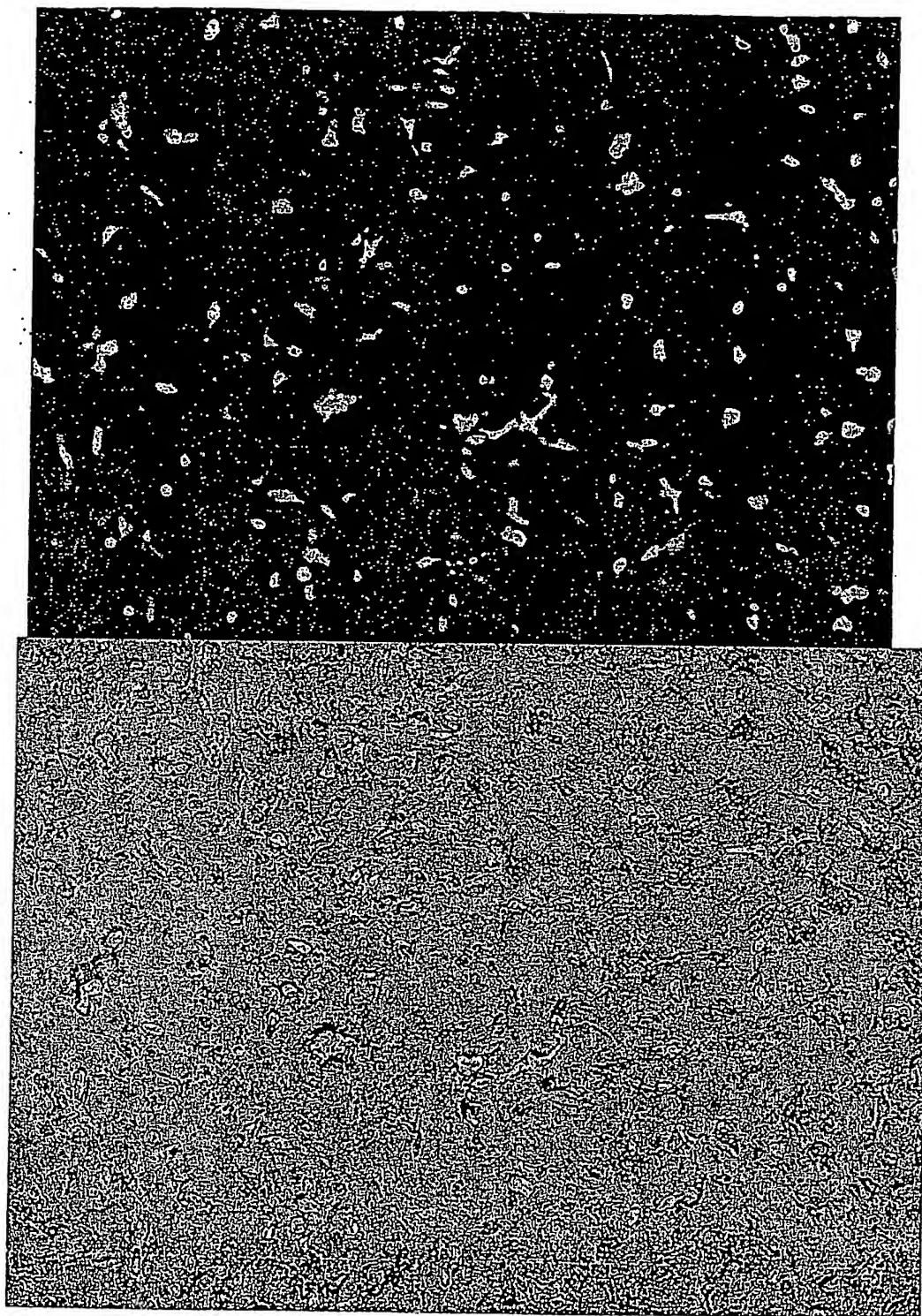


Fig. 9

Fig. 10



Transfection of NIH 3T3 cells with pGFP by B3A/DOPE 2:1

**Figure 11A**

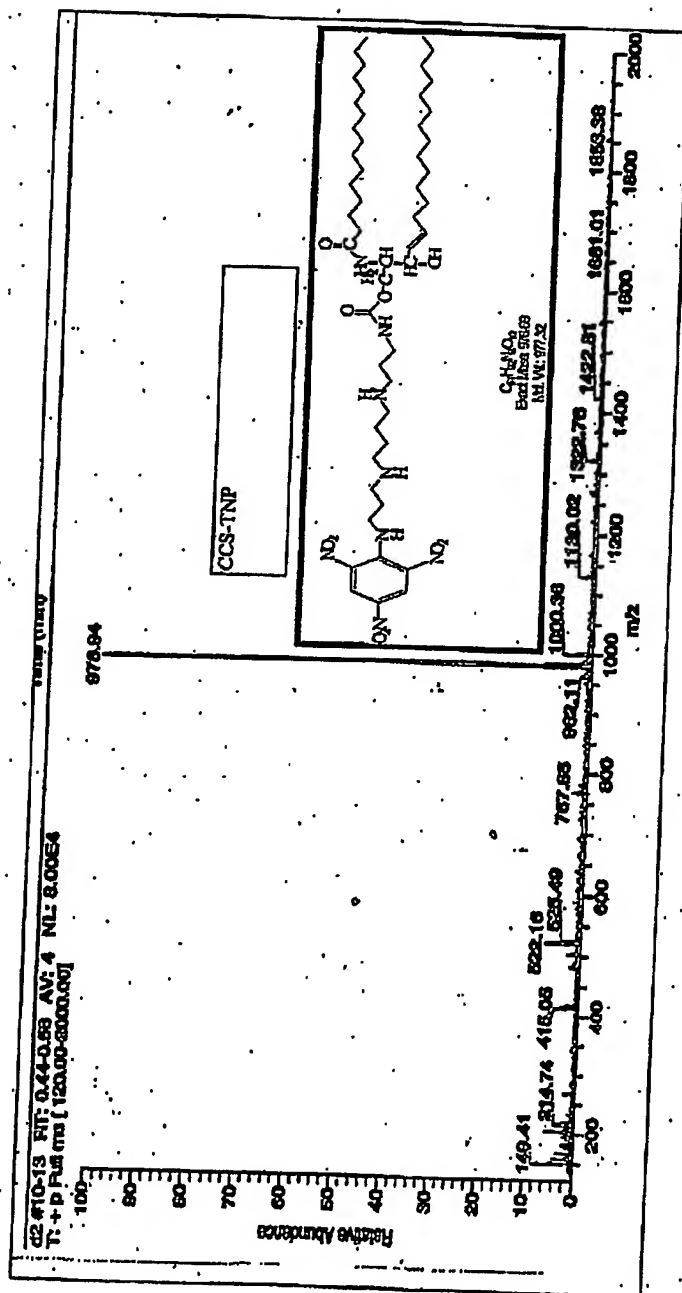
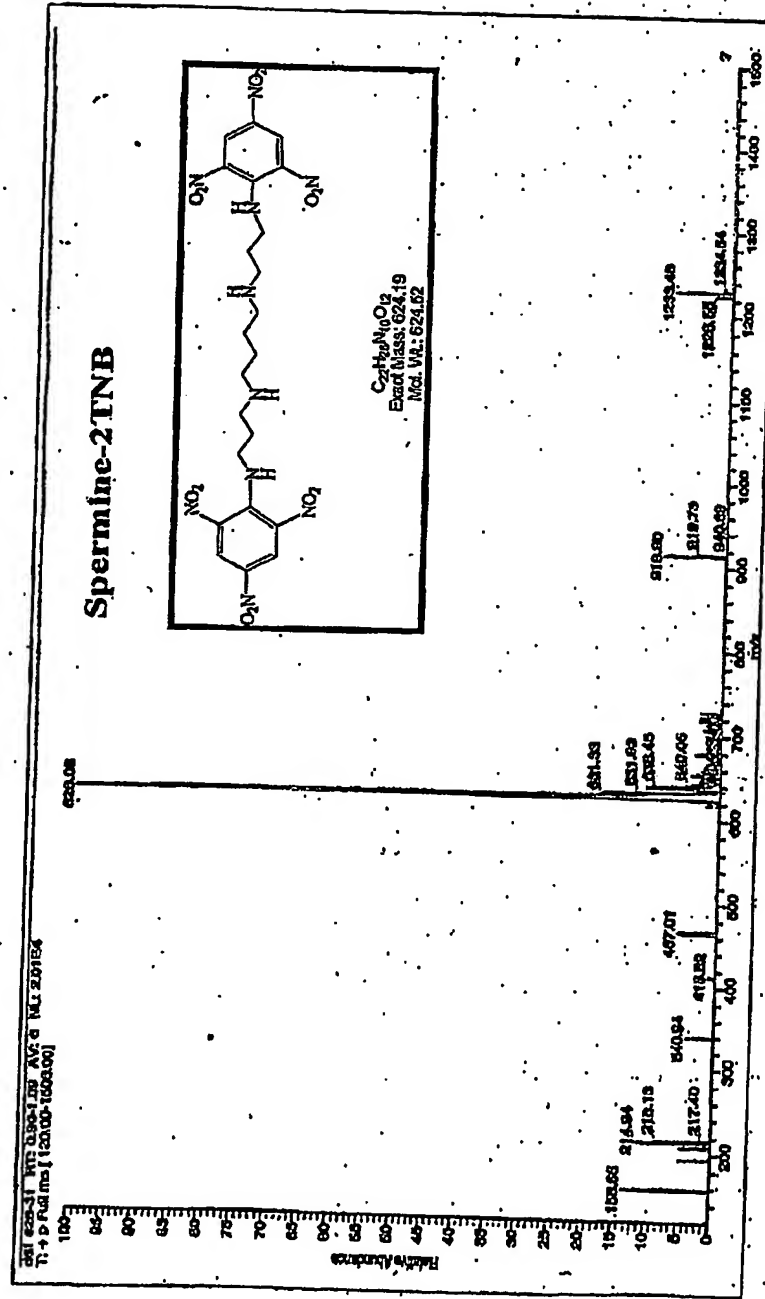




Figure 11B

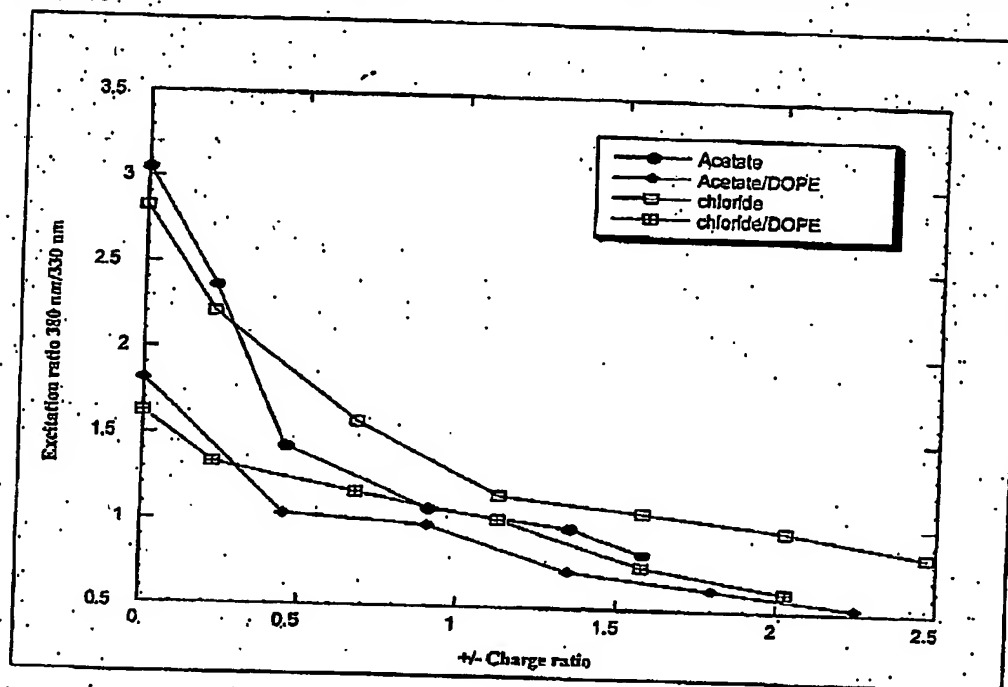




**Figure 12**

Titration of different liposomes with plasmid DNA

Titration was monitored by changes in bilayer-incorporated HC excitation curve upon addition of DNA



**Figure 13**

Hydration of different liposomes before and after addition of plasmid DNA  
Hydration was monitored by changes of Laurdan excitation General Polarization  
(the higher value means lower hydration)

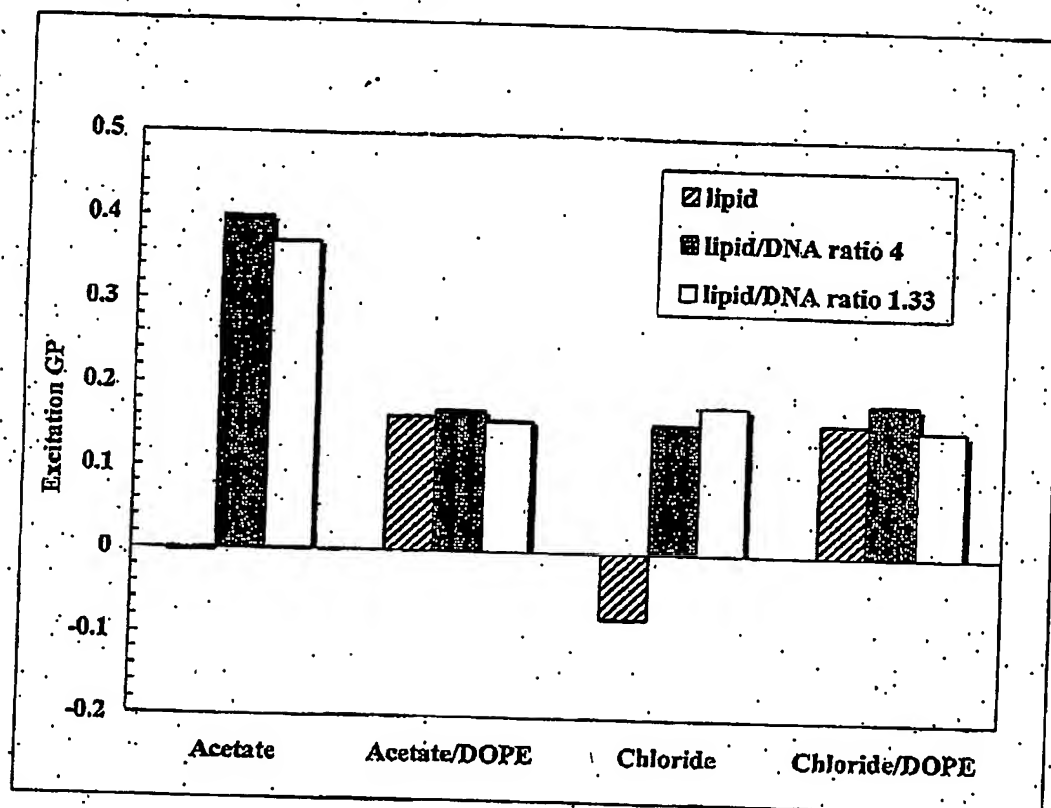


Figure 14A

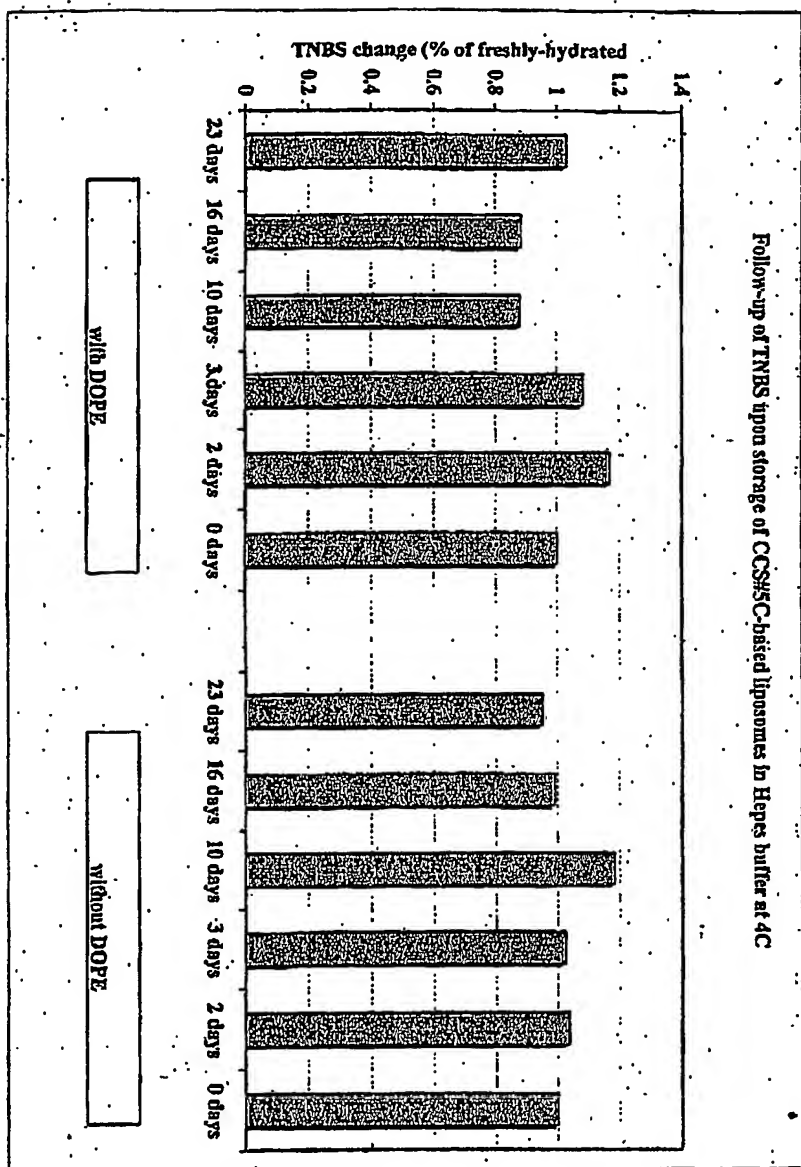
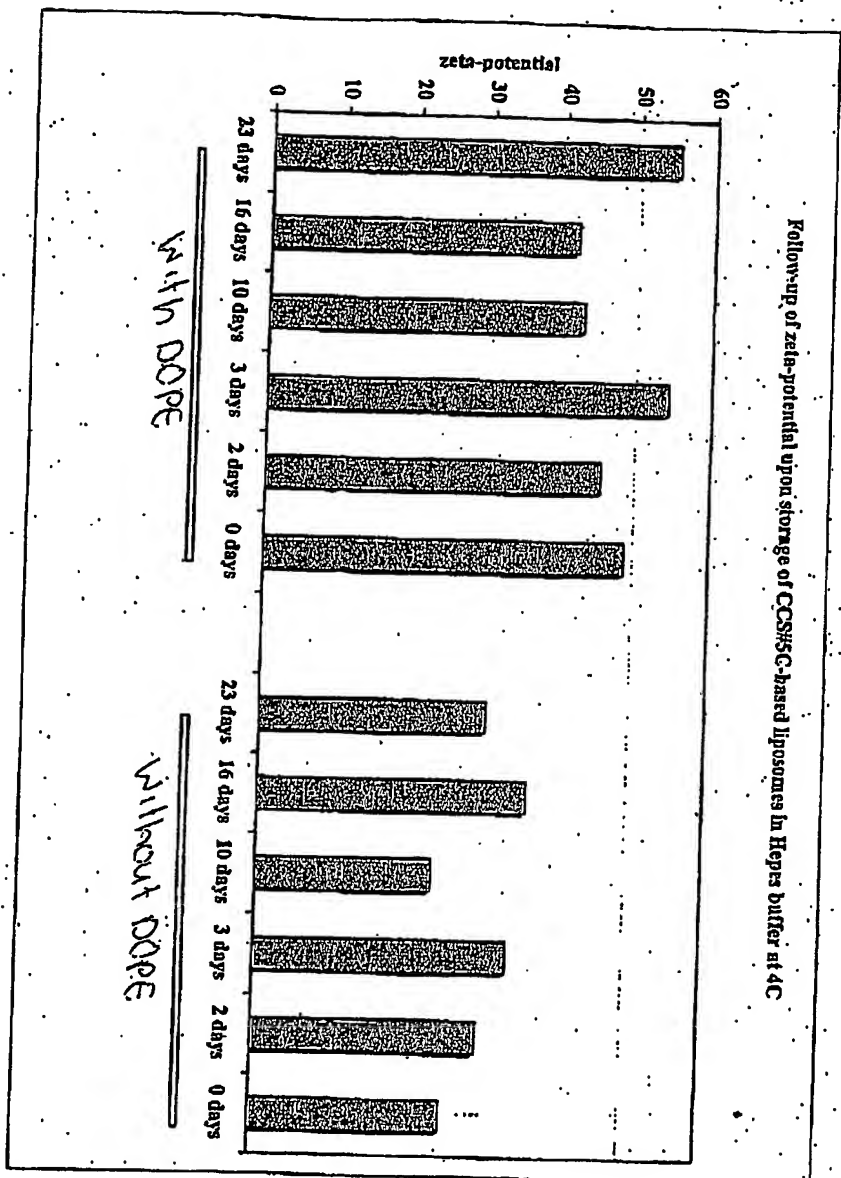


Figure 14B



**Figure 14C**

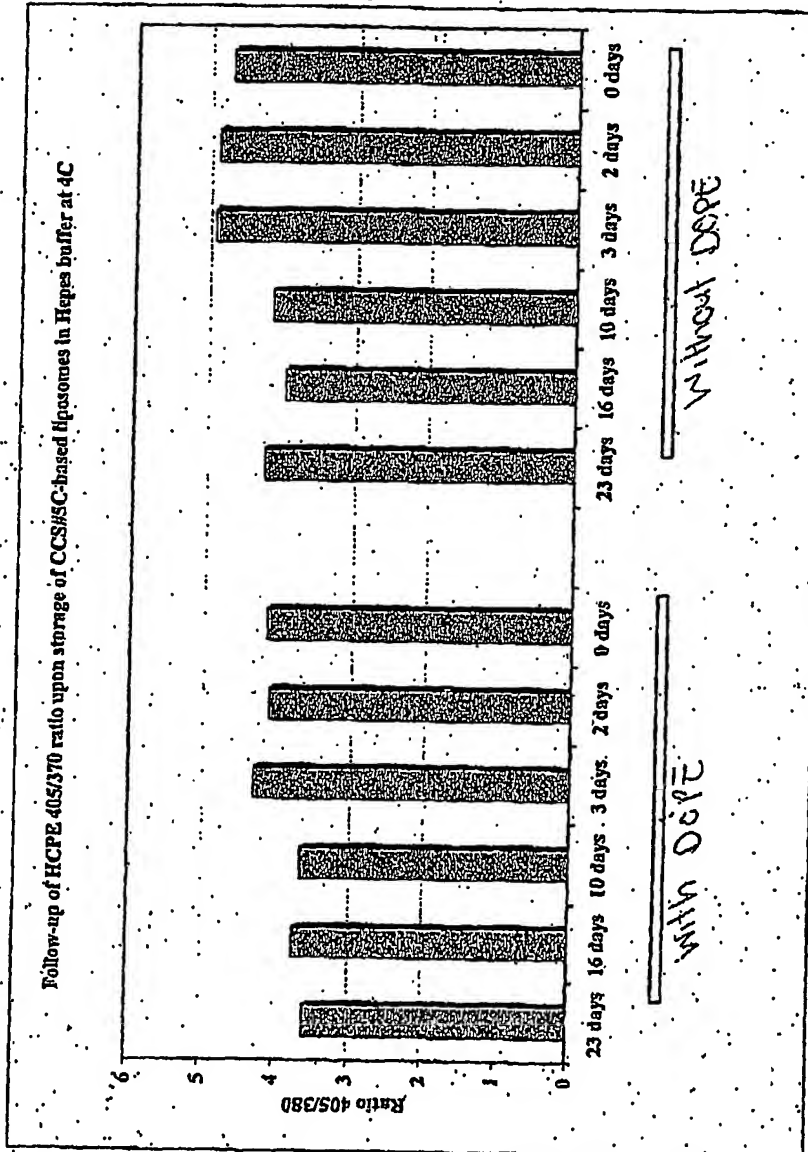
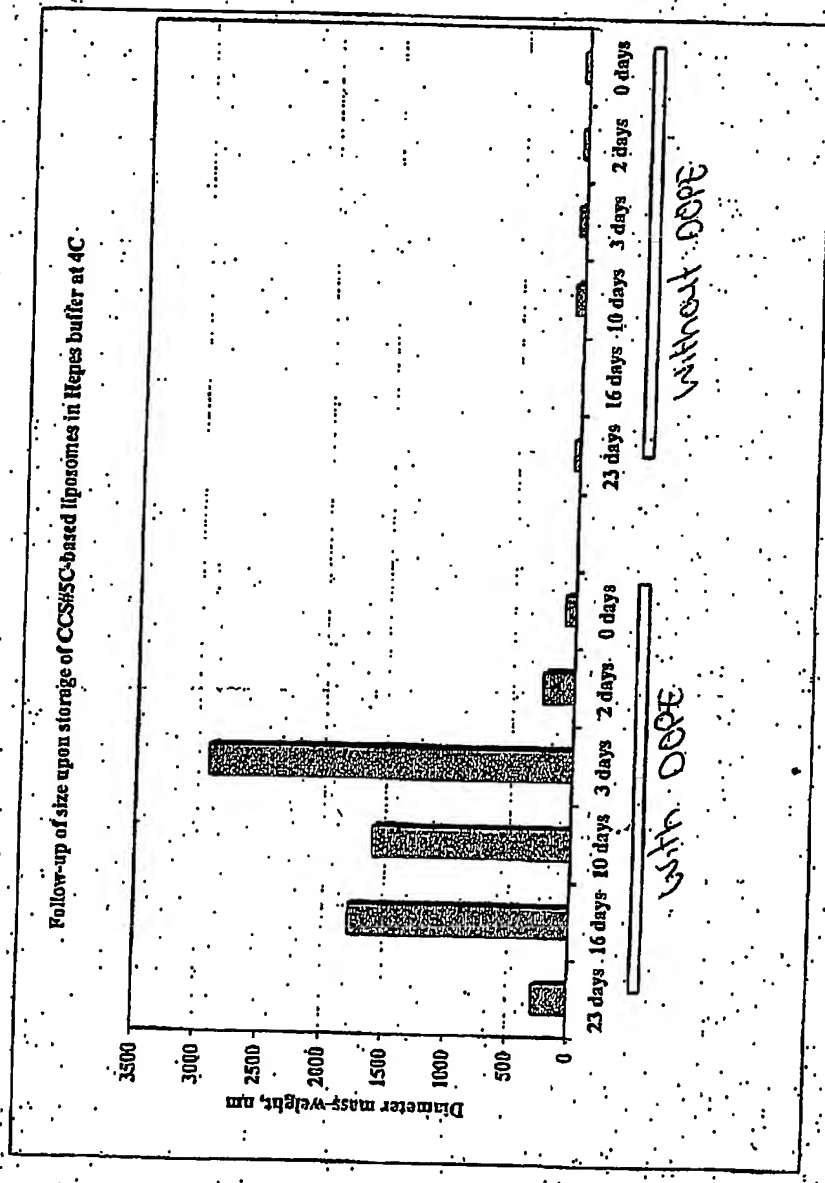
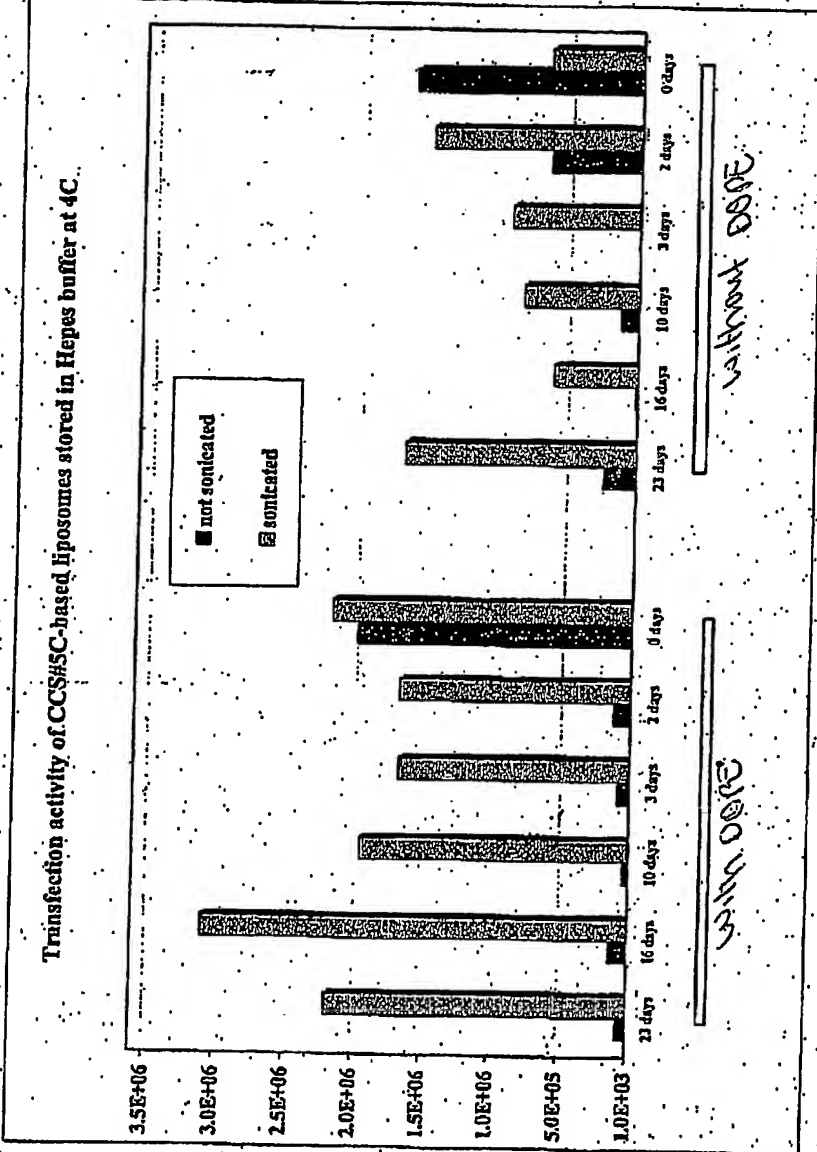


Figure 14D





**Figure 14E.**



**Figure 15A**

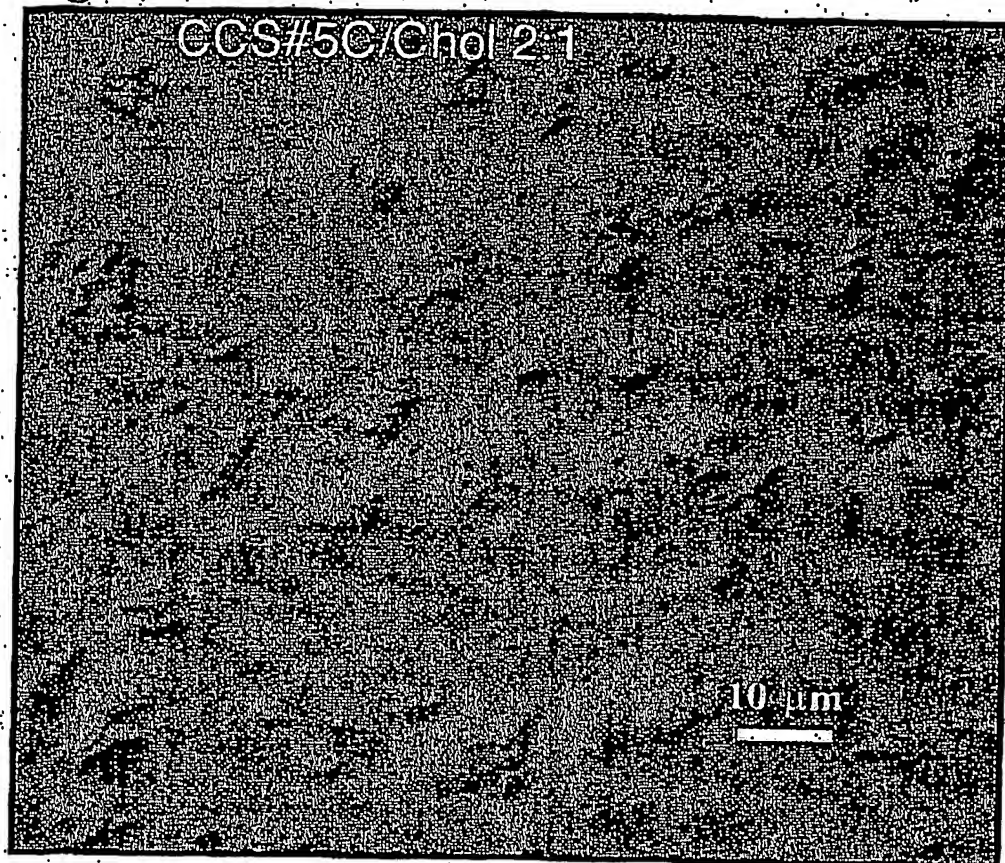


Figure 15B

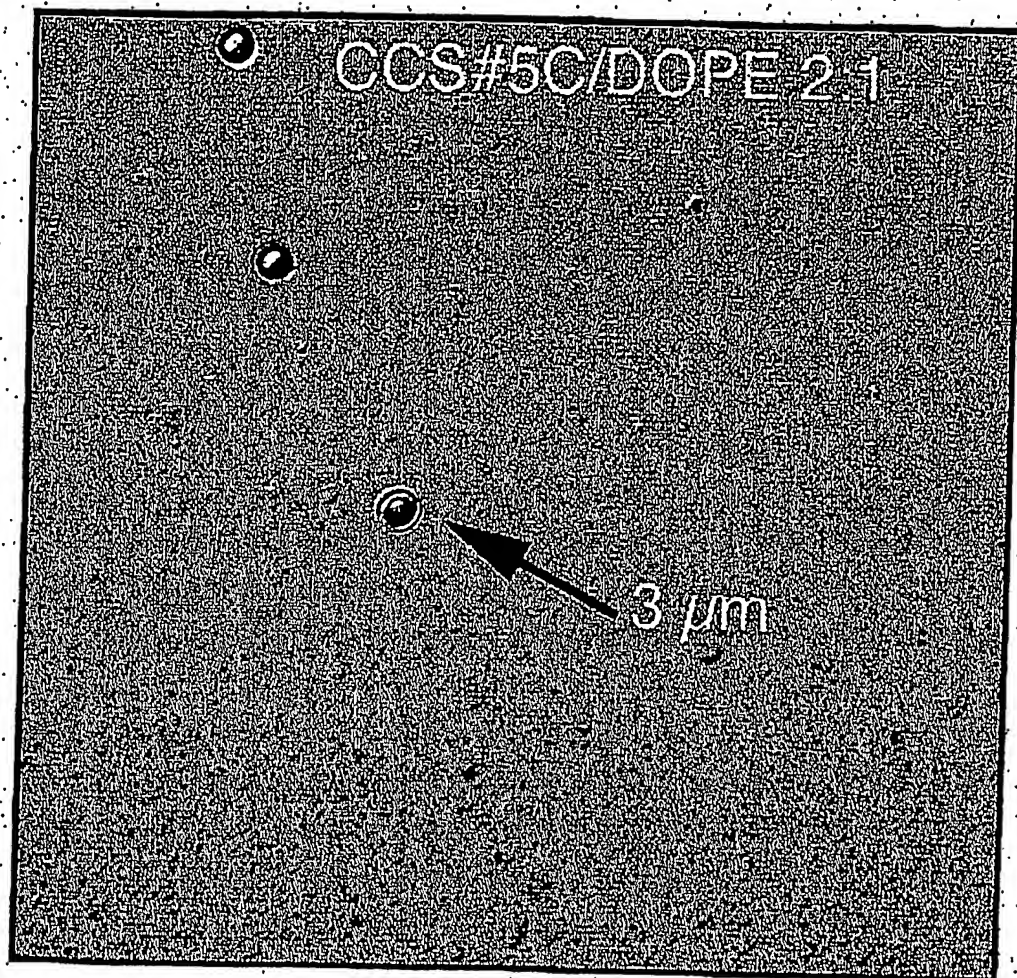
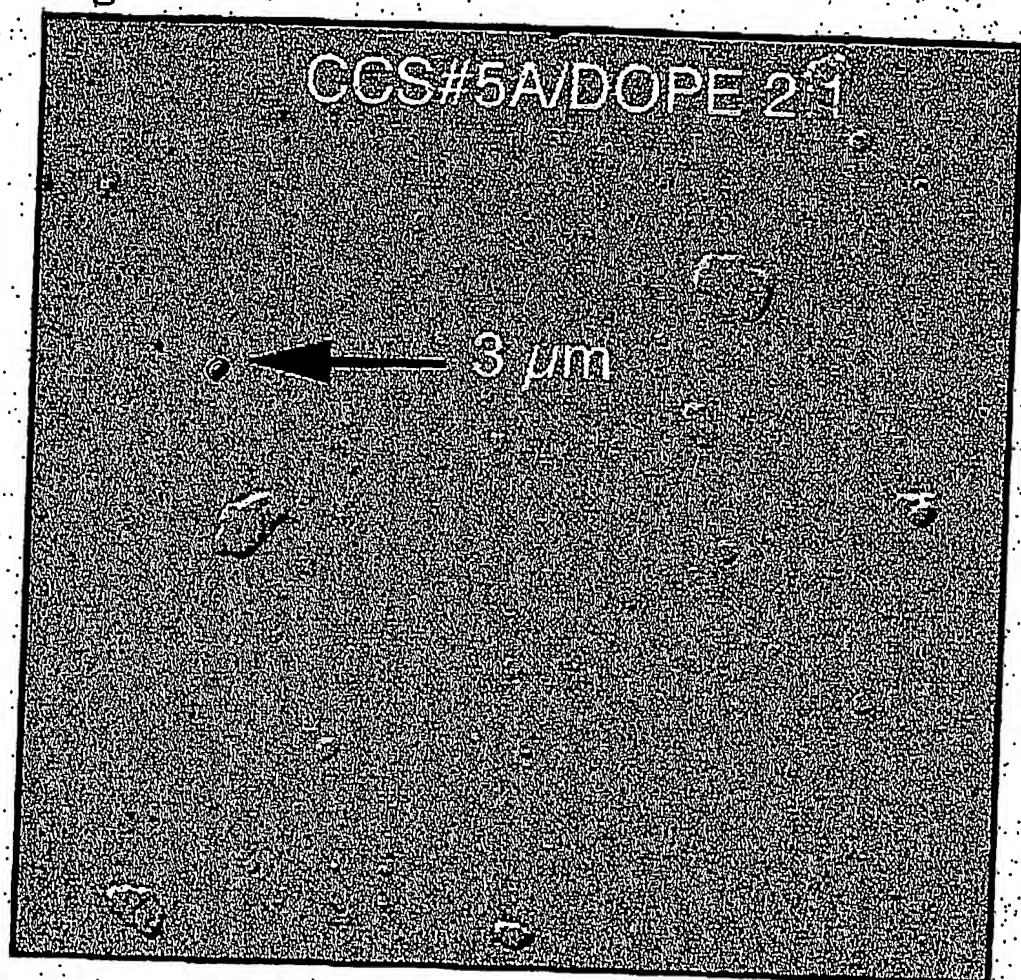
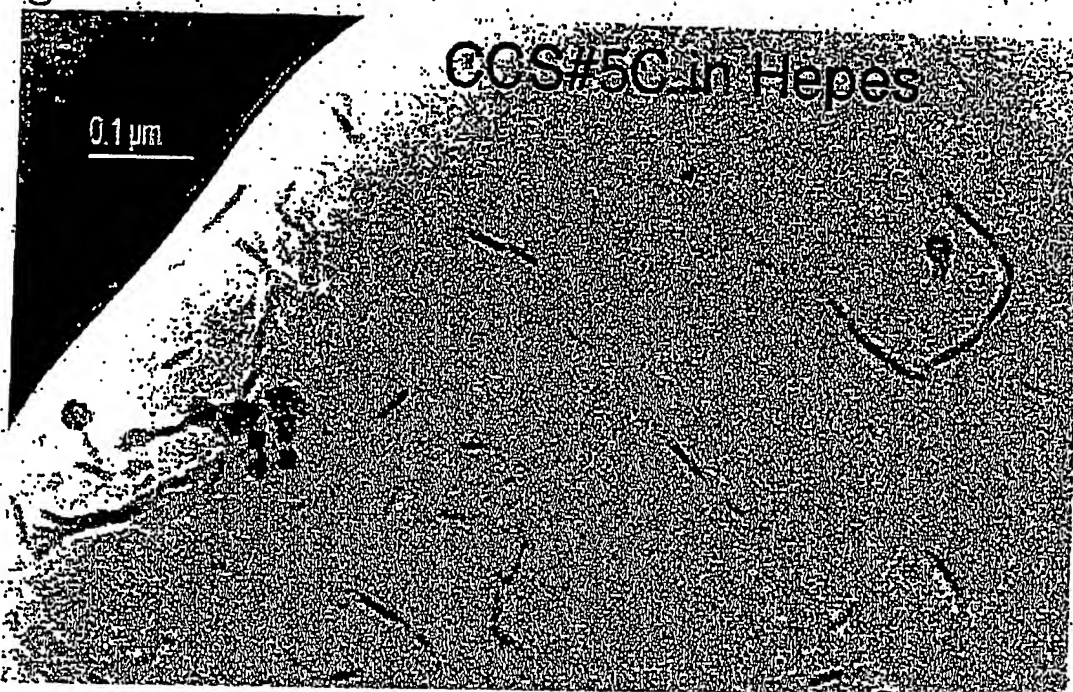


Figure 15C



**Figure 16A**



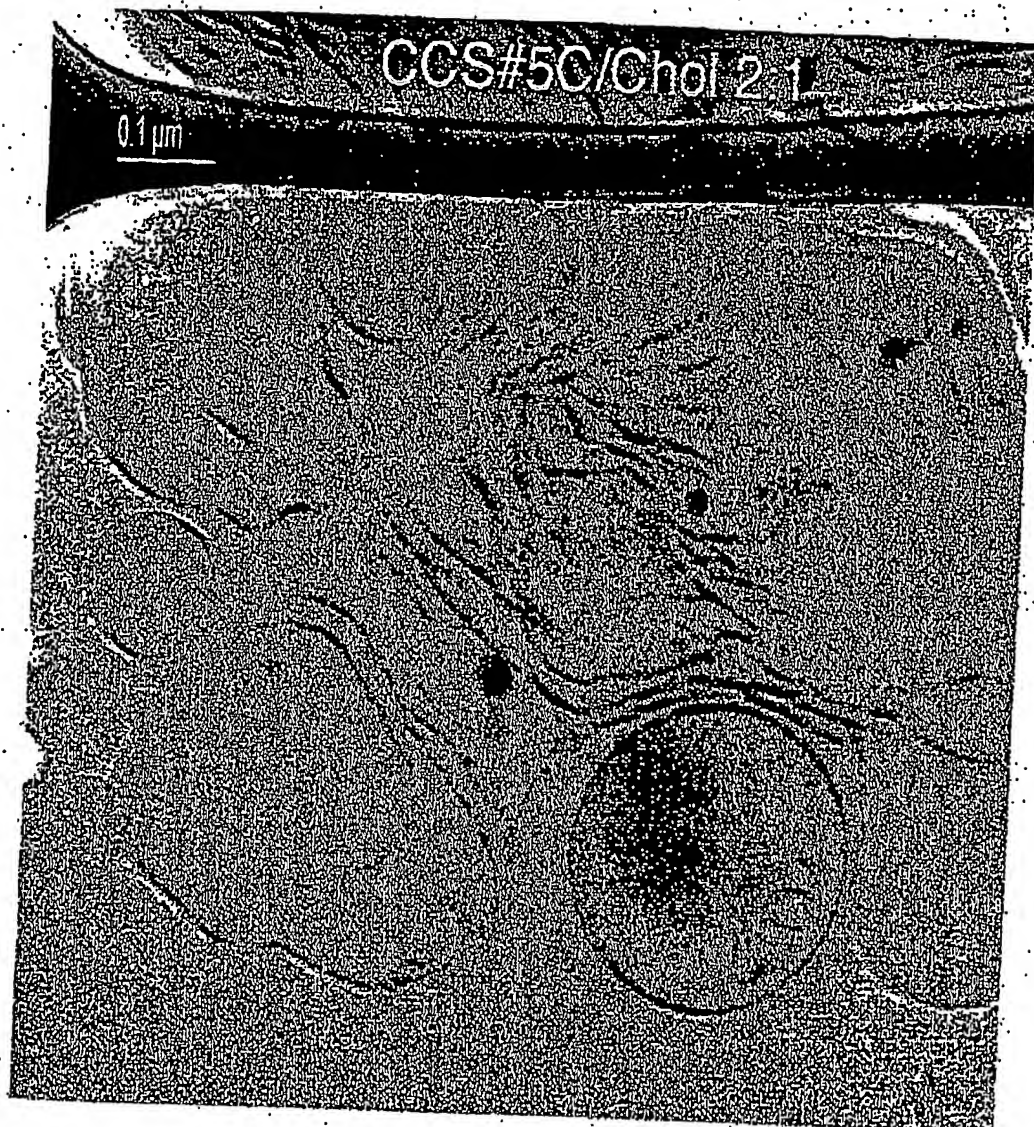


CCS#5C/DOPE 21

This electron micrograph shows a cell with a large, dark, electron-dense nucleus containing a prominent nucleolus. Several mitochondria with visible internal cristae are present in the cytoplasm. A scale bar in the bottom left corner indicates 1000 nm. The text 'CCS#5C/DOPE 21' is printed at the top of the image.

CCS#5C/DOPE 2-1

**Figure 16C**



**Figure 16D**

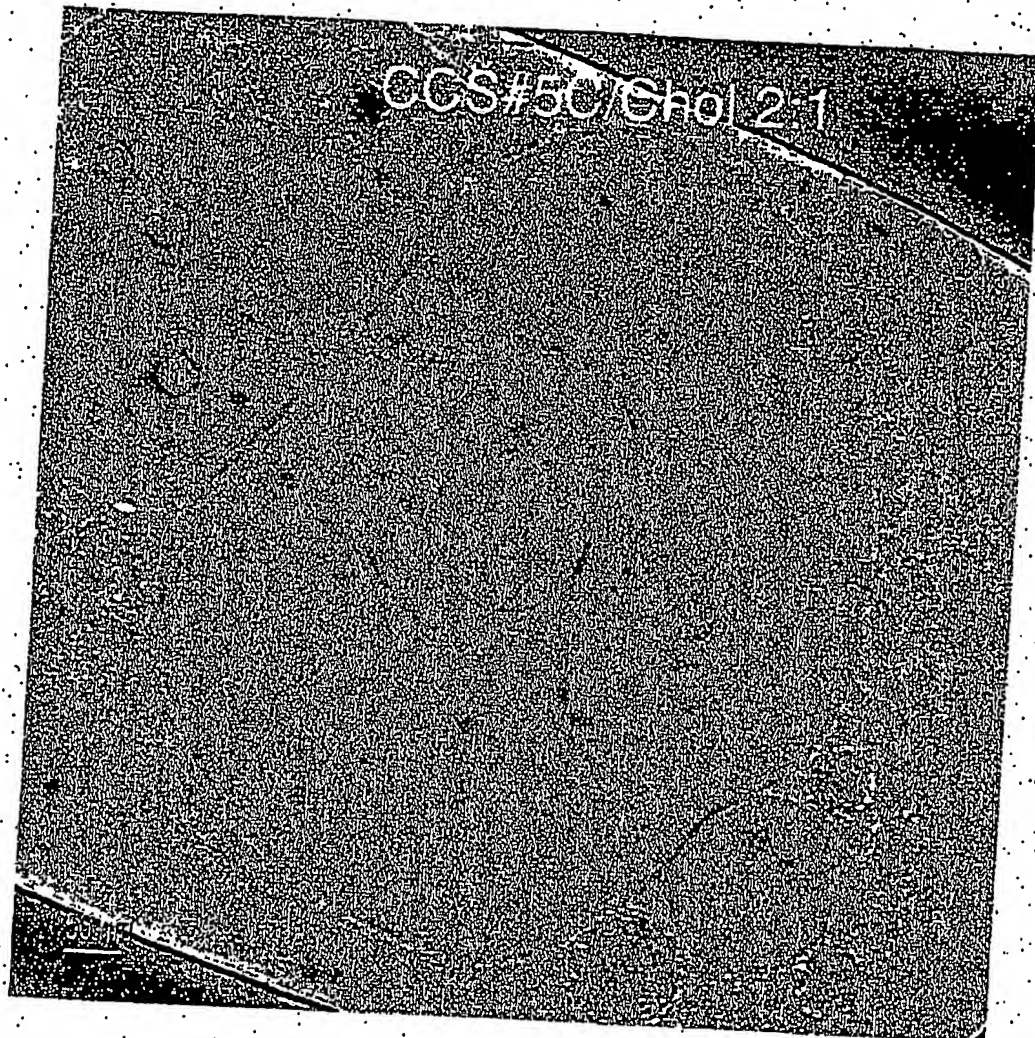




Table 1

Effect of the lipid (DOTAP)/protein (HN) ratio and cholesterol (Chol) on HN encapsulation efficiency

DOTAP/HN w/w ratio	DOTAP/Chol mole ratio	% HN encapsulation
300/1	1/1	93
100/1	1/1	90
50/1	1/1	90
30/1	1/1	88
10/1	1/1	79
3/1	1/1	35
100/1	1/0	90
100/1	1/1	92
100/1	2/1	89
100/1	4/1	80

Table 2

Serum, lung and nasal levels of HI, IgG1, IgG2a and IgA antibodies, and IFN $\gamma$  levels produced by spleen cells, following i.n. vaccination with HN-loaded neutral, anionic and cationic liposomes

Group (n=5)	Vaccine <sup>a</sup>	Serum	Lung			Nasal			Spleen
		HI	IgG1	IgG2a	IgG1	IgA	IgG2a	IgA	IFN $\gamma$ (pg/ml) <sup>b</sup>
1		0	0	0	0	0	0	0	1800
2	F-HN	0	55	0	0	0	0	0	1400
3	Lip (DMPC)-HN (Neutral)	3 $\pm$ 7 (0)	150	0	30	0	0	0	4200
4	Lip (DMPC/DMPG)-HN (Anionic)	6 $\pm$ 13 (20)	500	0	40	0	0	0	4000
5	Lip (DC-Chol:DOPE)-HN (CAT1)	18 $\pm$ 7 (0)	0	0	35	PBS	0	0	4900
6	Lip (DSTAP:Chol)-HN (CAT2)	28 $\pm$ 29 (40)	20	0	20	0	0	0	2300
7	Lip (DDAB: Chol)-HN (CAT3)	136 $\pm$ 32 (100)	100	0	80	0	30	0	3100
8	Lip (DOTAP: Chol)-HN (CAT4)	576 $\pm$ 128 (100)	15000	730	1050	170	180	30	8000
9	Lip (DMTAP: Chol)-HN (CAT5)	672 $\pm$ 212 (100)	30000	470	3000	30	80	25	7800
10	Lip (LLC: Chol)-HN (CAT6)	2368 $\pm$ 1805 (100)	30000	9000	30000	1900	300	50	10200
11	F-HN+CT (1 $\mu$ g)	1664 $\pm$ 572 (100)	55000	7000	10000	1800	120	45	5200

Legend to Table 2

<sup>a</sup> The vaccine (3µg HN) was administered i.n. (10 µl per nare) on days 0 and 7. All liposomes (Lip) were prepared at a lipid/protein w/w ratio of 300/1; the molar ratio of cationic lipid/cholesterol (Chol) or DOPE (groups 5-10) was 1/1. Serum HI titer was tested on individual samples starting at 1/10 dilution; the data show the mean  $\pm$  SD and % seroconversion (in parentheses). Antigen-specific isotypes were determined by ELISA on pooled samples starting at 1/20 dilution; the data show the means. Sera were tested 4 weeks and lung homogenates (pooled) and nasal washes (pooled) 6 weeks after the second vaccine dose.

<sup>b</sup> Spleen cells were incubated with the HN subunit preparation (10 µg/ml hemagglutinin) for 3 days. IFN $\gamma$  levels in cell cultures without antigen were subtracted. In all groups, IL-4 levels were <20 pg/ml.

Table 3

The effect of the lipid/HN w/w ratio on the immunogenicity of HN-loaded cationic liposomes (CAT4, CAT5, CAT6)

No.	Vaccine <sup>a</sup> (n=5)	Lipid/HN w/w ratio	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFN $\gamma$ (pg/ml)
1	F-HN		0	0	0	0	0	0	0	0	7430
2	Lip (DOTAP)-HN (CAT4)	300/1	496 $\pm$ 295 (100)	15000	450	0	40	600	85	30	9780
3		100/1	196 $\pm$ 119 (100)	5000	280	0	40	500	20	0	42220
4		30/1	36 $\pm$ 50 (80)	1000	200	0	30	250	35	0	20440
5		10/1	28 $\pm$ 18 (60)	600	30	0	20	250	0	0	20400
6		3/1	0	20	0	0	10	20	0	0	27780
7	Lip (DMTAP)-HN (CAT5)	300/1	388 $\pm$ 260 (100)	2500	250	0	0	5500	200	1200	Not done
8		100/1	208 $\pm$ 107 (100)	2200	600	0	0	7000	350	0	
9		50/1	130 $\pm$ 118 (80)	850	150	0	0	4500	250	0	
10		30/1	48 $\pm$ 71 (40)	450	0	0	0	1500	110	0	
11		10/1	24 $\pm$ 35 (40)	120	0	0	0	500	0	0	
12	Lip (LLC)-HN (CAT6)	300/1	560 $\pm$ 480 (100)	2000	1800	200	80	12500	3000	20000	
13		100/1	752 $\pm$ 504 (100)	6500	6000	0	80	7000	5500	65000	
14		50/1	272 $\pm$ 156 (100)	1900	700	0	40	5500	900	20000	
15		30/1	112 $\pm$ 125 (80)	650	400	0	0	1500	200	0	
16		10/1	52 $\pm$ 68 (40)	275	440	0	0	500	200	0	
17	F-HN+CT (1 $\mu$ g)	-	896 $\pm$ 350 (100)	30000	8000	120	80	45000	2250	3000	
18	F-HN+Lip (DOTAP) (CAT4)	300/1	864 $\pm$ 446 (100)	5000	1500	0	0	6000	500	1200	
19	F-HN+Lip (DMTAP) (CAT5)	300/1	320 $\pm$ 226 (100)	1900	400	0	0	3750	225	1500	
20	F-HN+Lip (LLC) (CAT6)	300/1	704 $\pm$ 525 (100)	30000	5000	500	80	35000	3000	80000	

<sup>a</sup> Data compiled from 2 separate experiments. In exp. 1 (groups 1-6), 3  $\mu$ g HN, and in exp. 2 (groups 7-20), 2  $\mu$ g HN was administered twice i.n. (10  $\mu$ l per nares). In groups 18-20 the free antigen was mixed with performed liposomes in saline 30 min before administration. All liposomes were made with Chol at a cationic lipid/Chol 1:1 mole ratio. For details on assays, see Legend to Table 2.

Table 4

The effect of cholesterol (Chol) on the immunogenicity of HN-loaded CAT4, CAT5 and CAT6

N	Vaccine <sup>a</sup> o. (n=5)	Cat lipid/ Chol w/w ratio	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFN $\gamma$ (pg/ml)
1	F-HN	-	0	0	0	0	0	0	0	0	7430
2	Lip (DOTAP)-HN (CAT4)	1/0	320 $\pm$ 0 (100)	15000	450	0	40	900	85	25	7480
3	Lip (DOTAP:Chol)-HN	1/1	496 $\pm$ 295 (100)	15000	450	0	40	600	80	30	9780
4		2/1	168 $\pm$ 216 (100)	7000	800	0	40	680	180	22	12870
5		4/1	195 $\pm$ 111 (100)	15000	250	0	60	720	50	60	9330
5	Lip (DMTAP)-HN (CAT5)	1/0	320 $\pm$ 188 (100)	20000	290	0	60	1000	40	0	8520
7	Lip (DMTAP:Chol)-HN	1/1	672 $\pm$ 419 (100)	30000	300	0	120	3000	30	15	10900
3		2/1	576 $\pm$ 368 (100)	25000	650	0	160	2500	160	200	8560
3		4/1	608 $\pm$ 382 (100)	30000	600	0	80	4000	100	150	7490
3	Lip (LLC)-HN (CAT6)	1/0	2560 $\pm$ 1568 (100)	30000	7000	100	640	30000	1500	9000	15550
1	Lip (LLC:Chol)-HN	1/1	2368 $\pm$ 1805 (100)	30000	9000	100	1280	30000	1900	15000	13780
2	F-HN+CT (1 $\mu$ g)	-	1664 $\pm$ 572 (100)	55000	7000	20	20	10000	1800	1000	11110

<sup>a</sup> All liposomes were prepared at a lipid/HN w/w ratio of 300/1. The vaccine (3  $\mu$ g HN/dose, 10  $\mu$ l per nare) was administered i.n. on days 0, 7. For details on assays, see legend to Table 2.

Table 5

The effect of the instillation volume on the immune response induced by i.n. administration of HN-CAT5

Vaccine <sup>a</sup> (n=5)	Volume μl/nare/ dose	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFN <sub>γ</sub> (pg/ml)
PBS	10	0	0	0	0	0	0	0	0	20
F-HN	10	0	60	0	0	0	0	0	0	460
Lip (DMTAP:Chol, 1/1)-HN	5	180±123 (100)	7000	65	0	0	180	15	0	1240
	10	1248±1208 (100)	40000	1500	150	80	20000	150	700	18000
	25	1024±572 (100)	35000	800	35	80	1500	180	420	2700
	50	544±215 (100)	31000	325	0	80	1500	30	85	49000

<sup>a</sup> The vaccine was given on days 0, 7; each dose was divided between nares. For details on assays, see legend to Table 2.

Table 6  
The effect of free and liposomal CpG-ODN on the immunogenicity of free and liposomal HN

No.	Vaccine <sup>a</sup> (n=5)	Serum HI	IgG1	IgG2a	IgA	Lung HI	IgG1	IgG2a	IgA	Spleen IFN $\gamma$ (pg/ml)
1	F-HN	0	50	0	0	0	0	0	0	460
2	F-HN+CpG-ODN	30±60 (20)	480	150	0	0	28	30	0	2000
3	F-HN+Lip (DMPC/DMPG) CpG-ODN	164±148 (80)	5000	1480	45	40	90	150	32	4940
4	F-HN+Lip (DMTAP/Chol) CpG-ODN	960±905 (100)	28500	6750	90	80	3100	700	280	13600
5	Lip (DMTAP/Chol)-HN	1248±1208 (100)	40000	2500	150	80	25000	150	700	18000
6	Lip (DMTAP/Chol)-HN+CpG-ODN	1088±893 (100)	30000	4500	150	60	1800	900	700	13000
7	Lip (DMTAP/Chol)-HN+	448±175 (100)	36000	600	40	60	1000	150	120	1440
	Lip (DMTAP/Chol) CpG-ODN									
8	Lip (DMTAP/Chol)-HN+CpG-ODN]	1240±945 (100)	28000	8000	45	80	1000	7000	700	10400
9	F-HN+CT (1 $\mu$ g)	1340±730 (100)	35000	3000	190	20	3500	500	1000	12000

<sup>a</sup> HN (2  $\mu$ g/dose), free and liposomal, was administered i.n. (10  $\mu$ l/nare/dose) on days 0, 7. All liposomes were prepared at a 300/1 (w/w) lipid/HN ratio; DMPC/DMPG, 9/1 mole ratio (anionic liposomes); DMTAP/Chol, 1/1 mole ratio (CAT5). In group 8, HN+CpG-ODN were co-entrapped within the same liposomes. In all groups, CpG-ODN (ODN 1018) dose was 10  $\mu$ g. For details on assays, see legend to Table 2.

Table 7  
Induction of cellular responses by cationic liposomes administered i.n.

No.	Vaccine <sup>a</sup>	Lipid/HN w/w ratio	% Cytotoxicity (Mean) <sup>b</sup> P815+peptide	P815	Proliferation <sup>c</sup> Δcpm (mean)
1	PBS		6 <sup>c</sup> /5 <sup>d</sup>	4 <sup>c</sup> /4 <sup>d</sup>	7009/3493 <sup>g</sup>
2	F-HN		8/4	5/4	7704/6959
3	CAT5-HN	300/1	16/8	13/7	10963/9119
4		100/1	9/4	9/4	12870/7353
5		50/1	3/2	3/2	17676/14831
6		30/1	3/0	2/0	17913/13742
7	CAT6-HN	300/1	4/0	2/1	20370/17624
8		100/1	21/13	7/6	24869/22014
9		50/1	6/5	3/3	20984/13010
10		30/1	8/7	5/4	11510/9699
11	F-HN+Empty CAT4	300/1	17/13	4/2	19392/15803
12	F-HN+Empty CAT5	300/1	17/13	7/6	11847/9519
13	F-HN+Empty CAT6	300/1	16/11	8/6	19269/15792
14	F-HN+CT (1μg)		10/5	9/3	28580/20858

<sup>a</sup> Mice were immunized on days 0 and 7. Splenocytes were harvested 6 weeks after the second vaccine dose. All liposomal formulations (groups 3-13) were made with Chol at a cationic lipid/Chol 1/1 mole ratio. In groups 3-10 the antigen was encapsulated in the liposomes; in groups 11-13 the free antigen was admixed with preformed empty liposomes.

<sup>b</sup> Measured in a 4h <sup>51</sup>Cr release assay at effector/target cell ratios of 100/1<sup>c</sup> and 20/1<sup>d</sup>. P815 were pulsed with the H2-HA2 189-199 peptide as described in the Materials and Methods.

<sup>c</sup> Splenocytes were incubated with 5 μg<sup>f</sup> or 0.5 μg<sup>g</sup> antigen per well for 72 h then pulsed with <sup>3</sup>H-thymidine for 16 h.



Table 8

Induction of humoral and cellular responses and protective immunity by HN-loaded cationic liposomes administered i.m. (x1), i.n. (x 1 or 2), or orally (x2)

No.	Vaccine <sup>a</sup> (n=5)	Route	Serum (n=10) HI	IgG1	IgG2a	IgA	Lung (n=5) HI	IgG1	IgG2a	IgA	Nasal (n=5) IgA	Spleen (n=5) $\Delta$ cpm (mean) <sup>b</sup>	IFN $\gamma$ (pg/ml) <sup>c</sup>	Lung (n=5) Virus titer (log 10) <sup>d</sup>
1	PBS		0	0	0	0	0	0	0	0	0	1641	0	7
2	F-HN	i.m. x 1	60±37 (70)	1000	40	0	0	80	0	0	0	1909	0	4
3		oral x 2	0	0	0	0	0	0	0	0	0	2253	0	ND
4		i.n. x 1	0	0	0	0	0	0	0	0	0	669	0	ND
5		i.n. x 2	0	55	0	0	0	70	20	0	0	2813	0	5
6	Lip (CAT4)-HN (DOTAP/Chol)	i.m. x 1	424±141 (100)	21000	5500	0	40	900	500	0	0	3452	3300	0
7		oral x 2	0	0	0	0	0	0	0	0	0	0	1150	ND
8		i.n. x 1	40±28 (50)	450	80	0	0	50	20	0	0	482	1900	ND
9		i.n. x 2	409±172 (100)	25000	1300	60	120	10000	1000	350	45	8391	3200	0
10	Lip (CAT5)-HN (DMTAP/Chol)	i.m. x 1	768±211 (100)	24000	8000	0	20	900	150	0	0	5632	0	1
11		oral x 2	0	0	0	0	0	0	0	0	0	553	0	ND
12		i.n. x 1	10±10 (0)	300	60	0	0	35	20	0	0	1277	0	ND
13		i.n. x 2	532±763 (100)	10500	380	50	240	20000	700	2200	60	7331	3150	0
14	Lip (CAT6)-HN ( /Chol)	i.m. x 1	864±1100 (100)	25000	10000	0	60	3500	900	0	0	6196	5750	0
15		oral x 2	0	0	0	0	0	0	0	0	0	476	550	ND
16		i.n. x 1	34±50 (20)	1000	30	0	0	120	0	35	0	1705	6250	ND
17		i.n. x 2	2289±1576 (100)	25000	20000	400	360	30000	5000	20000	120	4912	15500	0
18	F-HN+CT (1 $\mu$ g)	i.n. x 2	756±650 (100)	21000	15000	20	240	22000	2500	1800	50	1933	5650	0

<sup>a</sup> Mice were immunized once or twice (days 0, 7) using 1, 2 and 4  $\mu$ g HN/dose for i.m. (in 30  $\mu$ l), i.n. (10  $\mu$ l/nare) and oral (in 50  $\mu$ l) administration, respectively. All liposomes were prepared at a lipid/HN w/w ratio of 300/1 with Chol at 1/1 mole ratio. Sera were tested separately (HI) or pooled (Elisa) at 4 weeks, and lung, nasal and spleen responses and protective immunity (all pooled) at 6 weeks post-vaccination.

<sup>b</sup>  $\Delta$ cpm = (mean cpm with antigen, 2.5  $\mu$ g/well) - (mean cpm without antigen).

<sup>c</sup> IFN $\gamma$  levels produced by spleen cells incubated alone were subtracted. 0 denotes <20 pg/ml.

<sup>d</sup> Tested 4 days after infection. See Materials and Methods for details. ND, not done.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☒ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**